University of Alberta

Isolation and characterization of potentially pathogenic *Escherichia coli* present during beef production

by

Adam Blair Olson ©

A thesis
submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agriculture Food and Nutritional Science

Edmonton, Alberta

Spring 2003

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Abstract

Potentially pathogenic *Escherichia coli* strains were isolated on vancomycin cefixime cefsuludin washed sheep blood agar plates and characterized by polymerase chain reaction (PCR) for the presence of enterohaemorrhagic *E. coli* (EHEC) virulence genes. Enrichment broths from a group of faecal, hide, caecal and ground beef samples from a research cattle herd were screened by PCR for the presence of EHEC virulence genes. Seventy-five potentially pathogenic strains of *E. coli* were isolated that possessed a variety of virulence genes. The virulence gene *ehxA* was most commonly amplified. Of the 210 enrichment broths screened by PCR, 193 were positive for at least one virulence gene. There were 15 different combinations of PCR-amplified virulence genes detected in the samples. This study confirms that strains of *E. coli* with EHEC virulence genes are prevalent in cattle at all stages of production and processing. New methods for the isolation of EHEC are needed to ensure all EHEC are detected.

Acknowledgements

I would like to take this opportunity to thank my supervisor Dr. Lynn M. McMullen, for all of her assistance and guidance throughout my Master's project. I would like to especially thank her for the help that she gave me in preparing this thesis.

I would also like to thank my supervisory committee, especially Dr. G. G. Greer and Dr. Frances Nattress for all the help they provided while I was doing research at the AAFC Lacombe Research Centre. I would also like to thank the technical staff of AAFC-LRC, especially Lynda Baker, Brian Dilts and Debbie Olsen. They provided invaluable assistance and friendship during my time in Lacombe.

I would like to thank Dr. Ole Sorenson for performing PFGE analysis, and Dr. K. Magor for allowing me to do DNA sequencing in her laboratory.

I would like to thank the staff and graduate students of the food microbiology lab that were there for me in many ways. Helping with work, school and especially keeping me from losing my sanity during the long hours spent in the lab.

Finally I would like to thank my family, since without them I would never have been able to achieve this goal, especially my wife Christine Mesa for standing by me during all the ups and downs involved with finishing this degree.

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Abbreviations

AAFRD	Alberta Agriculture, Food and Rural Development
Ab	antibody
Ab-ppt	immunoprecipitation
A/E	attaching and effacing lesions
Ag	antigen
ATCC	American Type Culture Collection
BCIG	5-bromo-4-chloro-3-indoxyl-β-D glucuronide
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
CR-SMAC	cefixime-rhamnose sorbitol MaConkey Agar
CT-SMAC	cefixime-tellurite SMAC
DAEG	diffuselyl adherent *E. coli*
EaeA	intimin
EaggEC	enteroaggregative *E. coli*
EDAS	electorphoresis documentation and analysis system
EHEC	enterohaemorrhagic *E. coli*
EhxA	enterohaemolysin A
EIEC	enteroinvasive *E. coli*
ELISA	enzyme linked immunosorbent assay
EPEC	enteropathogenic *E. coli*
EOH	*E. coli* O157:H7 medium
ET	electrophoretic types
ETEC	enterotoxigenic *E. coli*
Gb3	globatriaosyceramide
GUD	β-glucuronidase enzyme
HC	haemorrhagic colitis
HUS	haemolytic uremic syndrome
IBDG	indoxyl-β-D-glucuronide
IMS	immunomagnetic separation
LEE	locus of enterocyte effacement
LMT	low melting temperature agarose
MLEE	multi-locus enzyme electrophoresis
MSA	modified sorbitol agar
mTSB-n	modified trypticase soy broth supplemented with novobiocin
MUG	4-methyllumbelliferyl-β-D-glucuronide
mQ H_2O	milli-Q water
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PFGE	pulsed field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
RDBH	reverse dot-blot hybridization
rfb	gene encoding the *E. coli* O157:H7 antigen
RPLA	reverse passive latex agglutination

AAFC-SRC	AAFC Pacific Agri-Food Research Laboratory culture
SDS	sodium dodecyl sulfate
SMAC	sorbitol MacConkey agar
SOR	sorbitol
STEC	Shiga-toxin producing *E. coli*
Stx-1	Shiga toxin 1
Stx-2	Shiga toxin 2
TBX	tryptone bile agar with X-glucuronide
TSA	tryptic soy agar
TSB	trypticase soy broth
*uid*A	non-functional β-glucuronidase gene of *E. coli* O157:H7
UPGMA	Unweighted pair group method using arithmetic averages
USDA	United States Department of Agriculture
UspA	universal stress protein of *E. coli*
VCCWBA	vancomycin, cefixime and cefsulodin washed sheep blood agar
VP	virulence profiles
WBA	washed sheep blood agar
WBMA	washed sheep blood agar supplemented with mitomycin c

1 Introduction

The microbial safety of fresh meats is of increasing concern to the food industry and consumers. In spite of proper sanitation practices, spoilage and pathogenic microorganisms will be inadvertently introduced onto the surface of meat and into ground meat during processing (Byrne et al., 2000). A number of foodborne illness outbreaks have been associated with the consumption of undercooked ground beef contaminated with *Escherichia coli* O157:H7. Cattle and other ruminants are thought to be the main reservoir for this strain of *E. coli.* Humans infected with *E. coli* O157:H7 can develop serious illnesses, including, haemorrhagic colitis and haemolytic uremic syndromes. The ability to cause illness has been associated with the expression of many virulence factors, the most important of which are thought to be Shiga toxins (Stx), intimin (EaeA) and enterohaemolysin (EhxA). It was originally thought that *E. coli* O157:H7 was the only strain of *E. coli* that had all of these virulence factors and thus was the only strain capable of causing serious illness in humans; however, recent contamination of meat by non-O157 enterohaemorrhagic *E. coli* (EHEC) has become a concern (Arthur et al., 2002). There are still many questions as to how ubiquitous these organisms are in the cattle population and in meat.

Isolation of *E. coli* O157:H7 has become relatively routine with the discovery of many phenotypic differences between this strain and other *E. coli* strains. These include the inability to ferment sorbitol and the lack of β-glucuronidase activity. Many methods to detect the presence of strains of EHEC with virulence genes in faecal and food samples are based on the polymerase chain reaction (PCR), but PCR analysis is only one step in the analysis of non-O157 EHEC. Methods are also needed that isolate non-O157 strains from bovine samples. There is currently no method available to isolate and differentiate all of the non-O157 EHEC present in cattle populations or in the resulting meat products. Many non-O157 EHEC have been reported to produce EhxA, and new procedures have been developed to isolate strains that produce EhxA. One method that has been described that has the ability to isolate EhxA-producing *E. coli* strains is the plating of samples onto vancomycin-cefixime-cefsulodin washed sheep blood agar (VCCWBA). This medium allows EhxA-producing *E. coli* strains to be differentiated from other *E. coli* stains.

The primary objective of this study was to evaluate the use of different methods to determine the prevalence of potentially pathogenic strains of *E. coli* at different stages of cattle production and processing. Cattle from a research herd at the Agriculture and Agri-Food Canada Research Centre in Lacombe, Alberta were the source of the samples tested in this research. Use of a herd of cattle from a research centre with slaughter facilities permitted careful tracking of animals throughout the production cycle and through processing to the final product. This would not be possible to do under commercial conditions of production and slaughter. The effectiveness of VCCWBA and PCR to isolate and characterize strains of *E. coli* with the virulence genes, *stx*-1, *stx*-2, *eaeA* and *ehxA* obtained from faecal, caecal, hide and ground beef samples was evaluated.

Two approaches were taken in this research, the first was to examine for the presence of the *stx*-2 and *ehxA* genes, and further isolate strains from those samples that tested positive. Strains were characterized by PCR for the presence of the four virulence genes.

E. coli strains that tested positive for the presence of virulence genes were further characterized by randomly amplified polymorphic DNA analysis and pulsed field gel electrophoresis, to test if strains isolated at different stages of production were genetically related. The second approach was to enrich for *E. coli* strains from faecal, hide, caecal and ground beef samples taken from different stages of the production continuum in modified tryptic soy broth supplemented with novobiocin. These enrichment broths were then screened by PCR for the presence of the *E. coli* specific *uspA* gene. All samples that were positive for the presence of *E. coli* were then examined for the presence of *ehxA*, *eaeA*, *stx*-1, and *stx*-2 virulence genes. This was done to identify how prevalent these *E. coli* virulence genes were throughout the cattle production continuum.

2 Literature Review

2.1 Foodborne disease:

The Centers for Disease Control and Prevention (CDC) in Atlanta reported that there are more than 200 diseases that are transmitted through food (Bryan, 1982). The causes of these foodborne illnesses include viruses, bacteria, parasites, toxins, metals and prions. In developed countries like Canada and the United States, the greatest threat of illness from consumption of foods is associated with bacterial pathogens. There are an estimated 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths each year where the pathogen has been identified, where as unidentified pathogens are responsible for 62 million illnesses, 265,000 hospitalizations, and 3,200 deaths (Mead et al., 1999). Illnesses caused by foodborne microbial pathogens range from mild forms of gastroenteritis to life-threatening sequela including neurological, hepatic, and renal syndromes (Fey et al., 2000).

In recent years, substantial progress has been made in preventing foodborne diseases. For example typhoid fever, extremely common at the beginning of the 20th century, is now almost forgotten in the United States. Similarly cholera, bovine tuberculosis and trichinosis have also been controlled in developed countries. However, many new foodborne pathogens have emerged. Among the first of these were infections in foods linked to non-typhoid strains of *Salmonella* spp. These bacteria were not isolated from foods before the 1940's, but their isolation rates have increased every decade since (Tauxe, 1997). In the last 20 years, other infectious agents have been either newly described or newly associated with foodborne transmission. *Vibrio vulnificus*, *Cyclospora cayetaensis* and *E. coli* O157:H7 are examples of newly described pathogens that are often foodborne (Mead et al., 1999; Tauxe, 1997). The foods contaminated with emerging pathogens usually look, smell, and taste normal. These foodborne pathogens share a number of characteristics. The pathogen often survives traditional preparation techniques; for example *E. coli* O157:H7 in ground meat can survive the gentle heating that a hamburger cooked to "rare" receives (Cieslak et al., 1997). Virtually all have an animal reservoir from which they spread to humans. These organisms do not cause illnesses in the animal that is the reservoir and therefore, public health concerns must now include apparently healthy animals (Tauxe, 1997). Contamination with these new foodborne pathogens eludes traditional food inspection, which relies on visual identification of foodborne hazards (Gill et al., 1998). These pathogens demand new control strategies that minimize the likelihood of contamination of food (Brown et al., 2000; Gill et al., 1999; Tompkin, 1990).

In Canada, it has been estimated that the meat industry sustains annual losses of 200 million dollars due to spoilage, while recalls result in annual losses of 500 million dollars due to product recall, loss of work productivity, legal costs, and medical expenses (Greer, 1993). The losses in the United States are even greater, which is demonstrated by a recall that occurred in July 2002 that involved the recall of 19 million pounds of ground beef as a result of the presence of *E. coli* O157:H7. This recall was the second largest ground beef recall in the United States due to the detection of *E. coli* O157:H7 in the meat. The largest recall due to *E. coli* O157:H7 was in 1997, when 25 million pounds of ground beef was recalled from Hudson Foods Ltd., due to the detection of *E. coli* O157:H7. There is no doubt that the control of bacterial pathogens associated with meat can have significant economic consequences for the meat industry.

Meat and most meat products are highly susceptible to microbial spoilage and if mishandled, the growth of foodborne pathogens is possible (Collins, 1997; McDonald and Sun, 1999). The exterior of the animal is soiled with microorganisms, manure and soil material (these three combined are known as "tag") (Van Donkersgoed et al., 1997; Bell 1997). Microorganisms can be acquired from the farm or during transport to the slaughter abattoir (Byrne et al., 2000; Gill 1999). The amount of microbial contamination that can be transferred to the carcass varies, and is a reflection of the hygienic practices during slaughter, skinning and the opening of the body cavity (Bolton et al., 2001; Byrne et al., 2000; Tewari et al., 1999). Direct or indirect contamination from the animal hides, gut contents or faecal material, or by equipment that has been previously contaminated results in transfer of microorganisms that lead either to spoilage of the meat or contamination of the meat with microbial pathogens (Gill et al., 1998; Van Donkersgoed et al., 1997).

Meat and poultry related foodborne illnesses are most often caused by six bacterial foodborne pathogens. These are *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli* O157:H7 (Tauxe et al., 1997). The CDC has estimated that these six pathogens are responsible for 2 to 2.5 million meat related illnesses and 1,200 deaths in the United States each year (Frenzen et al., 2000). In Canada, *E. coli* O157:H7 is the third leading enteric pathogen associated with foodborne related illnesses and in Canada and the United States it is the fifth leading cause of death due to foodborne pathogens (Cuff et al., 2000, Mead et al., 1999). In the United States it has been estimated by Mead et al. (1999) that *E. coli* O157:H7 causes approximately 73,000 illnesses and 500 deaths each year. The disease associated with *E. coli* O157:H7 can range from self-limiting diarrhea to haemorrhagic colitis (HC, bloody diarrhea) and haemolytic uremic syndrome (HUS) (Fey et al., 2000) and *E. coli* O157:H7 is a serious cause of foodborne disease. Strategies should be developed to control the presence of these potential pathogens in the food supply. Studies into the ecology of these bacteria should be done to better understand how it is transmitted in the food supply.

Although *E. coli* O157:H7 has been established as the cause of foodborne illness, other non-O157:H7 shiga-toxin producing *E. coli* (STEC) are now emerging as foodborne pathogens. It has been estimated that non-O157:H7 STEC account for 20 to 50% of the illnesses, hospitalizations and deaths that are attributed to *E. coli* O157:H7 in North America (Park et al., 1996, Acheson et al., 1994). Recent studies from Canada (Ramotar et al., 1995), United States (Park et al., 1996; Fey et al., 2000), Europe (Bitzan et al., 1993; Thomas et al., 1996), Argentina (López et al., 1989; Padola et al., 2002) and Australia (Goldwater and Bettelheim, 1994) all suggest that non-O157:H7 STEC infections are as prevalent, or more so, than *E. coli* O157:H7 infections. It has also been determined that almost all non-O157:H7 STEC strains involved in foodborne diseases have two accessory virulence factors, intimin (encoded by the *eae* gene), and enterohaemolysin A (encoded by the *ehx*A gene) (Fukushima et al., 2000; Fagan et al., 1999; Beutin et al., 1995). Strains of *E. coli* with *stx*-1, *stx*-2 or both, *eae* and *ehxA* genes have the ability to cause illnesses and have been classified as EHEC (Gyles et al., 1998; Sandhu et al., 1996; Gannon et al., 1993).

2.2 Virulence of EHEC:

The natural habitat of *E. coli* is the gastrointestinal tract of warm-blooded animals, and in humans. This species is the most common facultative anaerobe found in the gastrointestinal tract (Prescott et al., 1996). Although most strains exist as harmless symbionts, there are many pathogenic *E. coli* strains, including EHEC that can cause a variety of diseases in animals and humans. Pathogenic strains differ from the normal microflora due to the presence of genes that express virulence factors. These virulence factors are directly involved in pathogenesis and have no known function in normal cellular metabolism. Expressing these virulence factors, the bacterium disrupts the normal physiology of the host and elicits disease. Virulence factors not only have a role in the disease process, but they presumably enable the pathogen to exploit their hosts in ways unavailable to non-pathogenic strains, allowing the pathogen to spread and persist in the bacterial community (Zieburh et al., 1999). There are six categories of pathogenic *E. coli*: enterotoxigenic (ETEC), enteroaggregating (EaggEC), enteroinvasive (EIEC), diffusely adherent (DAEC), enteropathogenic (EPEC) and the group with the highest morbidity in developed countries EHEC (Acheson et al., 1998).

Virulence factors that distinguish EHEC from commensal and other pathogenic *E. coli* have been acquired through lateral transfer of genes via bacteriophage, plasmids, and large portions of genomes from other bacteria (Karch et al., 1999). This occurs by the transfer of pathogenicity islands. These are relatively large (greater than 10 kb) genetic elements that encode virulence factors. These pathogenicity islands are found only in genomes of pathogenic strains and these strains frequently have a guanine and cytosine content that differs from that of the rest of the *E. coli* genome, indicating that they have DNA from another species of bacteria (Law, 2000; Kaper et al., 1998a).

Shiga toxins (Stx), which are encoded by bacteriophage, are the primary virulence factors implicated in diseases caused by EHEC (Bertin et al., 2001; Law, 2000; Melter-Celsa and O'Brien, 1998). Shiga toxins are members of a family of bacterial cytotoxins produced by *Shigella dysenteriae* type 1 and STEC or EHEC (Melton-Celsa and O'Brien, 1998). EHEC produce two genetically related Shiga toxins, Stx-1 and Stx-2, but these are immunologically distinct (Melton-Celsa and O'Brien, 1998). Shiga toxin 1 is almost identical to the Stx of *Shigella*, where as Stx-2 is highly divergent from the Stx of *Shigella*. There are 11 known Stx-2 variants that have been implicated in human illnesses, the most common being Stx-2, Stx-2a and Stx-2b (Bertin et al., 2001). All Stx are divided into a highly conserved single A subunit (97 to 100% conserved) that is responsible for enzymatic activity of the toxin and a highly divergent pentamer of B subunits (57 to 100% conserved) responsible for the binding of the toxin to a glycolipid receptor (Globatriaosyceramide; Gb3) (Acheson et al., 1998; Melton-Celsa and O'Brien, 1998). The divergences in the B subunits are thought to be the reason for the 1000-fold higher toxicity to human endothelial cells of Stx-2 compared to Stx-1 (Law, 2000; Melton-Celsa and O'Brien, 1998). The higher toxicity of Stx-2 is also thought to be due in part to alteration of receptor binding preference or affinity (Lindgren et al., 1994), or to the presence of multiple Stx-2 variants in one organism (Bertin et al., 2001). The presence of Stx-2 is more often associated with the development of HUS (Paton and Paton 2002; Law, 2000). Once in the cell, the toxin targets the 28 sRNA, which is depurinated by the toxin at a specific adenine residue, causing protein synthesis in the cell to cease and the infected cell to die by apoptosis (Law, 2000; Melton-Celsa and O'Brien, 1998). This destruction of blood vessel endothelial cells is the cause of HC (Acheson et al., 1998), where as HUS is caused by the systemic spread of Stx and subsequent damage to renal microvascular endothelial cells (Monnens et al., 1998).

Although Stx are important in diseases caused by EHEC, other virulence factors are also required for EHEC to cause disease. The minimal requirements for pathogenicity of EHEC are thought to be the presence of Shiga toxins and the ability to adhere to the bowel mucosa (Law, 2000). Intimin is a 94 to 97 kDa outer membrane protein, which is an intestinal adherence factor, produced by all attaching and effacing (A/E) enteric pathogens including EHEC (Kaper et al., 1998b; McGraw et al., 1999). Intimin is encoded by the *eae* (*E. coli* attachment and effacement) gene, which is found on the locus of enterocyte effacement (LEE) pathogenicity Island (Law, 2000; Kaper et al., 1998b; Fey et al., 2000). Intimin has been grouped into five categories (α, β, γ, δ, ε) based on the amino acid sequences and antigenic differences (Kaper et al., 1998a). The γ subtype is the most common in *E. coli* O157:H7, where as β and ε are the most common intimin subtypes in EPEC (Kobayashi et al., 2001; McGraw et al., 1999). Variation of the different intimins is mostly in the last 25% (approximately 200 amino acids) of the C-terminus where there is only 49% identity, where as there is 94% similarity in the first 75% (704 amino acids) of the N-terminal sequence. These differences are in the binding region of the protein and may be adaptations for binding to different intestinal epithelial cells causing different disease pathology (Kaper et al., 1998a.).

There have also been differences detected in the level of secretion of LEE encoded factors among different strains grown in different media. Strains implicated in human illness have greater secretion of LEE encoded factors than strains isolated from bovine sources (McNally et al., 2001). The higher level of secretion of LEE encoded factors may account for the variation of infective dose among different EHEC strains.

There are many other putative virulence factors that have been found on the LEE pathogenicity island in EHEC strains. Along with the *eae* gene there is a gene encoding a translocated receptor (*tir*) for intimin. The Tir protein forms the receptor that allows intimin to attach to the host cells. Without proper transcription of the *tir* genes strains cannot form A/E lesions (Kaper et al., 1998b). Also on the LEE are *esp* genes (*espA*, *espB* and *espD*) that encode secreted proteins responsible for inducing the epithelial cells signal transduction events leading to A/E lesions (Kaper et al., 1998b). The other set of known genes on the LEE are genes that encode a type III secretion system (*esc* and *sep*) that is involved in the extracellular secretion of the proteins encoded by the *esp* genes (Law, 2000; Kaper et al., 1998a). Even though Stxs produce the outward manifestation of disease caused by EHEC, the presence of the LEE pathogenicity island is necessary for EHEC to become established in the host and to cause disease (Law, 2000)

The putative virulence factor that is most common is the enterohaemolysin (*ehxA*) gene. Enterohaemolysin A is a member of the family of RTX toxins, like leukocytotoxin and APX toxins, which are known virulence factors. Enterohaemolysin is a pore forming toxin that is very effective on bovine leukocyte cells. In one study, the *ehxA* gene was found in 95% (Karch et al., 1998) of EHEC strains isolated from humans. In another study, the *ehxA* gene was associated with 78 to 90% (Gyles et al., 1998; Fey et al., 2000) of EHEC isolated from people with serious illnesses and only 15 to 50% of EHEC isolated from patients with non-severe illnesses (Gyles et al., 1998). Due to this association with serious illnesses, EhxA has been suggested as a good marker to indicate the presence of the EHEC (Karch et al., 1999).

Putative virulence factors found on the large EHEC plasmid other than EhxA have not been as well studied. Of these an EHEC type II secretion system is encoded by *etpC* to *etpO* (EHEC type II secretion pathway) genes. These genes have been found to have similarities to various secretion pathways of gram-negative bacteria (Genin and Boucher, 1994; Brunder et al., 1999). The function and specificity of this putative transport system have not been fully elucidated. It is found in 100% of *E. coli* O157:H7 isolates, but only in 52% of non-O157 EHEC (Karch et al., 1998). A bifunctional catalase peroxidase (KatP) encoded by *katP* genes has also been identified, which may be produced to detoxify oxidants produced by macrophages and neutrophiles, although this process has not been fully described for EHEC. The *katP* genes are only found in 66% of *E. coli* O157:H7 and 38% of non-O157 EHEC (Karch et al., 1998). A secreted serine protease (EspP), which cleaves human coagulation factor V, has also been isolated from EHEC. Similar serine proteases have been established as putative virulence factors in *Shigella flexneri* (Brunder et al., 1999). EspP increases vascular permeability, degrades immunoglobulin G, inhibits serum bacteriocidal activity by breaking down complement activity and may influence the blood-clotting cascade. The most likely impact of EspP is to increase the symptoms associated with bloody diarrhea. The *espP* gene has been isolated from 66% of *E. coli* O157:H7 isolates and only 36% of non-O157 EHEC (Karch et al., 1998; Brunder et al., 1999).

This variation in prevalence of putative virulence factors shows that they are not uniformly distributed among different EHEC strains that are pathogenic to humans

(Brunder et al., 1999). Whereas the plasmid of classical non-sorbitol fermenting EHEC O157:H7 are homogeneous in their gene composition, nearly all-possible combinations of the plasmid-encoded factors have been described in non-O157 EHEC (Karch et al., 1998). In addition to the virulence genes encoded on the plasmids, the arrangements of the genes within the plasmids can vary. Sequences adjacent to *espP* and *katP* genes have been shown to be different in *E. coli* serotypes O157, O26 and O103 (Fey et al., 2000).

For EHEC and STEC, there are many virulence factors that are involved in the pathogenesis that is observed in humans. The mechanisms of pathogenesis is not completely understood, however it is known that Stx-1, Stx-2, EaeA and EhxA are produced by most strains of EHEC and STEC that cause serious illness in humans. There are many accessory virulence factors that may contribute to the seriousness of disease caused by specific strains of EHEC or STEC. It has also been demonstrated that many of these virulence factors are located on mobile genetic elements, which have converted formerly harmless strains of *E. coli* into strains that can cause serious illnesses.

2.3 Evolution of EHEC:

To survive, bacteria must conserve their genetic information from one generation to the next. However, bacteria live and survive under continuously changing environmental conditions and if they cannot adapt to these changing conditions they will die. Bacteria have evolved strategies to allow the generation of genetic diversity. Point mutations, recombination between homologous DNA sites, and the transfer of transposable genetic elements are mechanisms through which genome flexibility is achieved. The capture and spread of genes by horizontal gene transfer through plasmids, phages and other mobile elements also contribute to this process. Finally, the clustering of genes on large genomic islands (pathogenicity islands) and their mobilization enables bacteria to gain or loose large amounts of DNA involved in the adaptation to distinct ecological niches. This allows the rapid development of new bacterial variants. Once successfully adapted, microorganisms tend to stabilize the newly generated genotype by adaptive mutations and it is thought that they can maintain it for millions of years (Zieburh et al., 1999).

Multi-locus enzyme electrophoresis (MLEE) has been used to estimate the genetic relatedness of closely related strains of EHEC and STEC (Pupo et al., 1997, Whittam, 1998). Multi-locus enzyme electrophoresis separates allelic variations of closely related enzymes on the basis of differences in the rate of their electrophoretic migration. To estimate the genetic relatedness among isolates, electromorphs can be equated with alleles at the corresponding enzyme locus and electrophoretic types (ETs) with multi locus genotypes. It is assumed that isolates with the same ET owe their genotypic similarity to recent descent from a common ancestral cell; that is, they are members of a naturally occurring bacterial clone (Strockbine et al., 1998; Whittam, 1998).

A study of 76 strains of *E. coli* O157:H7 from epidemic and sporadic cases of HC and HUS in North America by Whittam (1998) revealed that the 76 strains were divided into three ETs with the majority of the strains in one ET. *E. coli* O157:H7 does not cluster closely with any other STEC and is only distantly related to a second set of EHEC (EHEC2), which includes *E. coli* serotypes O111:NM and O26:H11 (Whittam, 1998). The use of MLEE revealed that *E. coli* O157:H7 is closely related genetically to an EPEC strain of serotype O55:H7, a non-Stx producing clone associated with infantile diarrhea. This suggests that the immediate ancestor of *E. coli* O157:H7 was a pathogenic clone with an ability to acquire novel virulence factors in nature (Donnenberg and Whittam, 2001).

An evolutionary model has been proposed by Feng et al. (1998) that describes the possible steps that have occurred in the emergence of *E. coli* O157:H7. The model is based on the assumption that during divergence, the probability of loss of function greatly exceeds gain of function for metabolic genes, and that gain of function usually occurs via the lateral transfer of genes. Also the sequence of events invoking the fewest changes would be the most likely model. The findings of Feng et al. (1998) are based on MLEE and are shown in Figure 1.

The model starts with the assumption that there is an ancestral EPEC-like strain that has the ability to ferment sorbitol and has β-glucuronidase (GUD) activity. This strain then acquires the LEE at the *selC* site and also acquires the A to T mutation in the *uidA* gene. It is thought that this A1 ancestral strain acquired the *stx*-2 gene, presumably by transduction by a toxin converting bacteriophage, resulting in an Stx-2 producing *E. coli* O55:H7 strain. From A2 to A3 the bacterium acquires the EHEC plasmid and *rfb* region (converting the O55 antigen to O157) and also the T to G +92 mutation occurs resulting in the A3 ancestral strain that is GUD negative, SOR positive and Stx-2 positive. This is where a split in evolution occurs with one stream losing motility and becoming A4 (O157:H- GUD+ SOR+ Stx-2+). The second stream of evolution looses the ability to ferment sorbitol and acquires the *stx*-1 gene, again through the transduction by a bacteriophage, thus giving rise to A5. Finally the loss of GUD activity occurs giving rise to clones of the modern *E. coli* O157:H7. Recent loss of *stx* genes and motility in

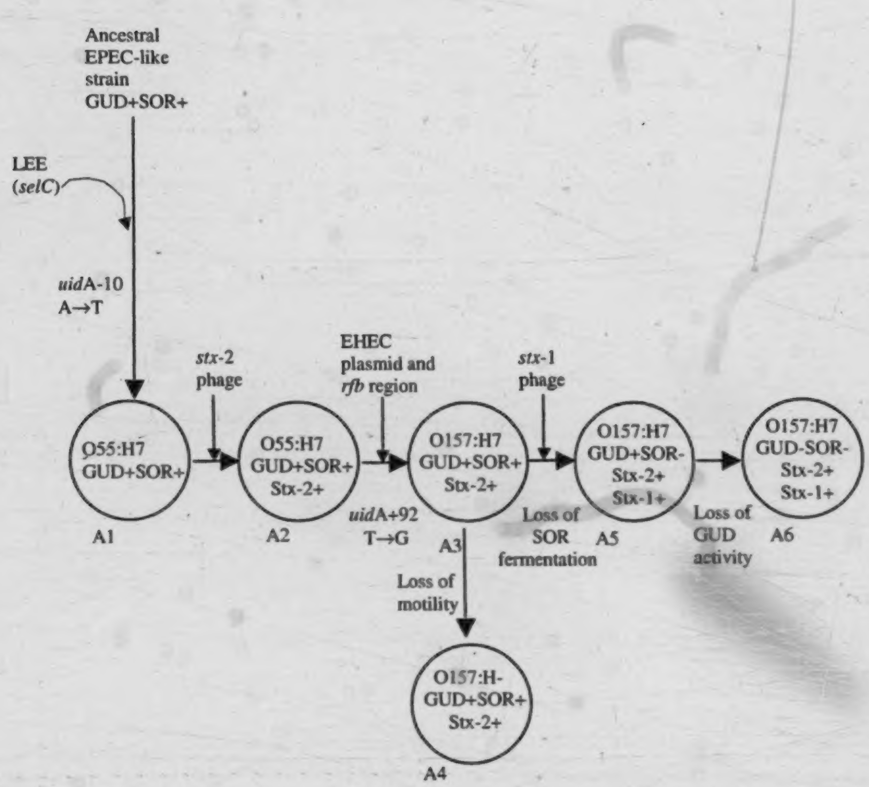
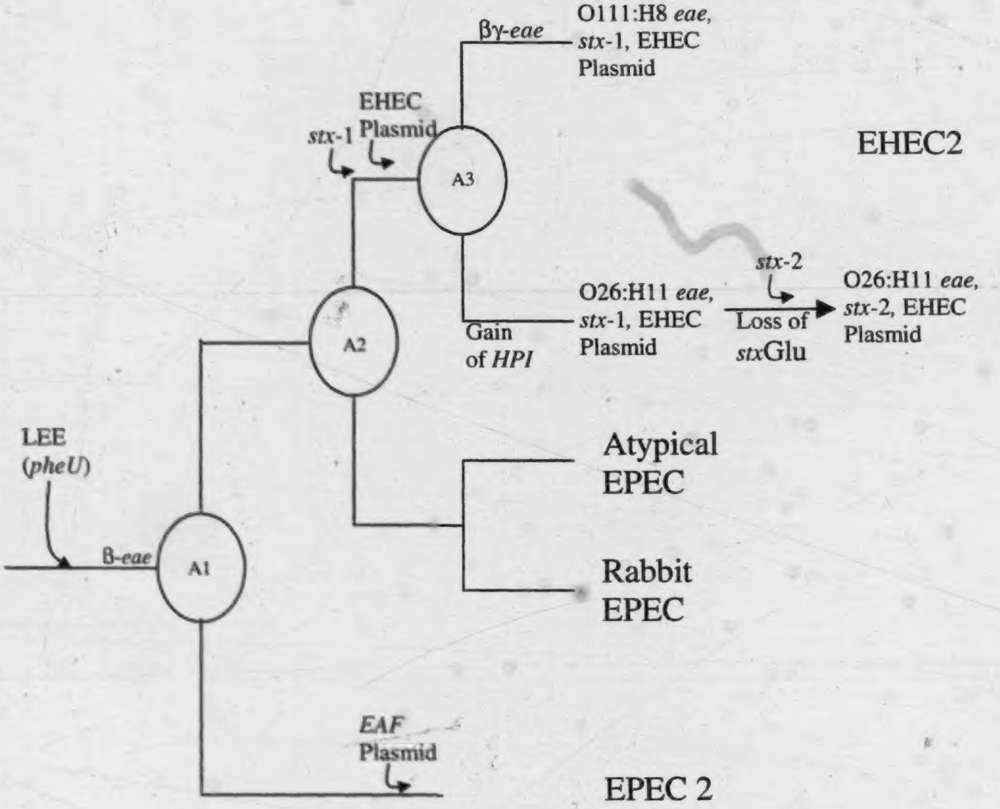

Ancestral
EPEC-like
strain
GUD+SOR+
LEE
(selC)
uidA-10
A→T
stx-2
phage
EHEC
plasmid and
rfb region
stx-1
phage
O55:H7
GUD+SOR+
A1
O55:H7
GUD+SOR+
Stx-2+
A2
uidA+92
T→G
O157:H7
GUD+SOR+
Stx-2+
A3
Loss of
motility
O157:H-
GUD+SOR+
Stx-2+
A4
Loss of
SOR
fermentation
O157:H7
GUD+SOR-
Stx-2+
Stx-1+
A5
Loss of
GUD
activity
O157:H7
GUD-SOR-
Stx-2+
Stx-1+
A6

Figure 1: The proposed stepwise evolution model for *Escherichia coli* O157:H7 from and ancestral EPEC-like strain based on the insertion of the LEE pathogenicity island at the *selC* gene. SOR, Sorbitol fermentation. GUD, β-glucuronidase activity. *E. coli* O157:H7, O antigen encoded by the *rfb* region. LEE, Locus of enterocyte effacement. Base mutations in the *uidA* gene at nucleotide position –10 (A to T), and at position +92 (T to G). Stx, Shiga toxin. (modified from Whittam, 1998)

nature or during isolation and culturing would account for the variants among isolates of the clones characterized to date (Whittam, 1998).

Much less is known about the virulence properties, epidemiology and evolution of the EHEC2 group, the most commonly isolated group of non-O157 Stx-producing strains. Although they often have serotypes O111:H8, O111:H-, O26:H11 or O26:H-, members of EHEC2 include diverse O:H serotypes, and many of these strains are nonmotile or cannot be typed using standard antisera. Because members of this group have the same principal virulence factors as *E. coli* O157:H7 (i.e., Stx and LEE) and are recovered from patients with HC and HUS, they have been classified together with *E. coli* O157:H7 as EHEC. However, evolutionary genetic analysis indicates that this group is sufficiently divergent from *E. coli* O157:H7 (Reid et al., 2000) to be considered as a second group of EHEC (Whittam and McGraw, 1996).

Donnenberg and Whittam (2001) have suggested a stepwise model to explain the evolution of this second group of EHEC. The emergence of this pathogenic lineage is thought to begin with the acquisition of the LEE pathogenicity island, which is found at the *pheU* site instead of the *selC* locus in the *E. coli* chromosome. This different insertion site is conserved in EPEC2 and EHEC2. This ancestral LEE carried a β intimin gene, which is found among the diverse serotypes in this group. From this stage, the group diverged into the EPEC2, with the EAF plasmid, and the EHEC2 group. Subsequent steps in the evolution of EHEC2 have not yet been fully elucidated but apparently involved multiple gains and losses of *stx* genes and pathogenicity islands. This model can be seen in Figure 2; however, these are just suggestions by the



βγ-eae
O111:H8 eae, stx-1, EHEC Plasmid
EHEC Plasmid
stx-1
A3
EHEC2
stx-2
O26:H11 eae, stx-1, EHEC Plasmid
Loss of stxGlu
O26:H11 eae, stx-2, EHEC Plasmid
Gain of HPI
A2
LEE (pheU)
ß-eae
A1
Atypical EPEC
Rabbit EPEC
EAF Plasmid
EPEC 2

Figure 2: Proposed evolution of the second group of EHEC with insertion of the LEE pathogenicity island at the *pheU* gene instead of the *selC* gene. Branch lengths are arbitrary and not set to an evolutionary scale. Gains and losses of genes or phenotypes are marked below the branches. HPI, high pathogenicity island. LEE, Locus of enterocyte effacement. *Eae*, gene encoding intimin. Stx, Shiga toxin. (adapted from Donnenberg and Whittam, 2001).

Donnenberg and Whittam (2001) and they have not yet been confirmed as the actual evolutionary steps that occurred in the emergence of EHEC2. It is known that there have been a number of recombinations within the intimin gene, giving rise to a mosaic of combinations seen within this group. Also the so-called "high pathogenicity island" (HPI) from pathogenic *Yersinia* has been very recently acquired in the O26 lineage (Donnenberg and Whittam, 2001). This is an example of the extreme plasticity of the *E. coli* genome, having the ability to acquire virulence factors by horizontal gene transfer and to develop certain strains that are inherently more pathogenic than others.

EHEC strains represent an impressive example of development of a pathogen by lateral gene transfer. Gene transfer processes lead to a mosaic pattern of pathogenicity factors as observed in many EHEC (Perna et al., 1998). Enterohaemorrhagic *E. coli* strains are a heterogeneous group of pathogenic organisms with respect to their serotypes, *stx*-1 and *stx*-2 genes and presence of additional virulence factors. With the use of molecular evolutionary genetic techniques, EHEC have been divided into two main groups (EHEC1 and EHEC2) that evolved independently of each other (Donnenberg and Whittam, 2001; Boerlin, 1999; Whittam, 1998). Enterohaemorrhagic *E. coli* 1 and 2 have been classified on the basis of the presence of multiple virulence traits that provide the distinctive pathologies associated with these organisms. The acquisition of these many virulence traits has provided an increased adaptation to new ecological niches allowing a greater chance of survival for these bacteria.

2.4 Cattle as a reservoir of EHEC:

Since the first outbreak of *E. coli* O157:H7 in ground beef in 1982, a bovine source has been implicated in many outbreaks (Riley et al., 1983, Wells et al., 1983). The intestinal tract of ruminants is currently considered as the main natural reservoir of *E. coli* O157:H7 and other STEC (Caprioli and Tozzi 1998). Numerous studies have reported isolation of *E. coli* O157:H7 from healthy beef and dairy cattle, showing that cattle are probably asymptomatic carriers (Wells et al., 1991; Hancock et al., 1994; Zhao et al., 1995). Faecal shedding of *E. coli* O157:H7 and non-O157 EHEC by cattle is intermittent, characterized by short periods of relatively high shedding followed by longer periods of reduced or undetectable shedding (Wells et al., 1991; Besser et al., 1997). Reported levels of *E. coli* O157:H7 shed by cattle have ranged from 10^2 to 10^5 CFU/g feces (Zhao et al., 1995, Wang et al., 1996).

Initial studies showed a low prevalence of *E. coli* O157:H7 in the faeces of cattle (Hancock et al., 1994; Faith et al., 1996). In a study by Hancock et al. (1994), the prevalence of *E. coli* O157:H7 in the faeces of dairy and beef cattle was 0.28% and 0.71%, respectively, with an overall prevalence in the herds of 8.3% and 16%, respectively. A more recent study by Chapman et al. (1997a) demonstrated that the prevalence of *E. coli* O157:H7 in the faeces of beef and cull dairy cattle was 15.7% (752 of 4800) over a one-year study of 400 cattle. The monthly prevalence ranged from 4.8% to 36.8% with the highest prevalence occurring in the late summer (Chapman et al., 1997a). This seasonal pattern may contribute to the similar seasonal pattern of *E. coli* O157:H7-associated foodborne illness in humans (Ostroff et al., 1989). Van Donkersgoed *et al.* (1999) isolated *E. coli* O157:H7 from 7.5% of the faecal samples collected from cattle just after slaughter, and the prevalence of *E. coli* O157:H7 in faecal samples from yearling cattle (12.4%) was higher than in samples from cull cows (2.0%). Even more recently, Elder et al. (2000) reported a prevalence of 28% (910 of 3270 animals) for *E. coli* O157:H7 in faecal samples taken from slaughter cattle, just prior to slaughter during July and August. Sampling done in the United States showed the prevalence of *E. coli* O157:H7 in feedlots to range from 63 to 100%, indicating widespread distribution of *E. coli* O157:H7 in cattle operations (Dargtz et al., 1997; Elder et al., 2000).

The increased prevalence of *E. coli* O157:H7 reported in recent studies is likely due to the use of more sensitive isolation methods compared to prior isolation using media alone. A 10- to 100-fold increase in the sensitivity to detect *E. coli* O157:H7 from bovine faecal samples has been reported through the use of immunomagnetic separation (IMS; Chapman et al., 1994a; Wright et al., 1994). Geographic location also has an impact on prevalence. A higher prevalence of *E. coli* O157:H7 has been reported in the northwest USA, Canada and northern England (Sheffield), than in other countries worldwide (Chapman, 2000). Other variables that could influence the prevalence of *E. coli* O157:H7 include season, type of feed, number of samples taken, frequency and timing of sampling, and how samples are transported and stored (Van Donkersgoed et al., 1999; Elder et al., 2000).

Carriage of non-O157:H7 STEC in cattle has been reported to be similar to that of *E. coli* O157:H7. In Argentina the prevalence of non-O157 STEC was found to be 16.3% (López et al., 1998). Montenegro et al. (1990) reported that in Germany the carriage of non-O157 STEC in cattle faeces was 11%, where as Burnens et al., (1995) found that the prevalence of non-O157:H7 STEC in cattle faeces from Switzerland was 21%. Smith et al. (1998) reported the highest prevalence of non-O157 STEC in cattle faeces with 34% of faecal samples from a beef cattle herd in the United Kingdom tested positive for non-

O157 STEC. The prevalence of STEC is usually based on the identification of Stxs alone, and does not usually include tests for intimin and enterohaemolysin, which are required for an *E. coli* strain to be considered as an EHEC.

Escherichia coli O157:H7 was first associated with human illness in 1982. When first discovered, *E. coli* O157:H7 prevalence was thought to be very low (<1%). As research has increased and detection methods have improved, reports on the prevalence of *E. coli* O157:H7 have increased. At first, *E. coli* O157:H7 was thought to be the only serotype important in causing disease. In the last 10 to 15 years, the prevalence of non-O157 STEC has been reported to be the same or higher than that of *E. coli* O157:H7 in many countries. It is important to find out what proportion of these non-O157 STEC possess all the virulence factors necessary to cause disease in humans and to determine the prevalence of these bacteria in cattle and meat.

2.5 Prevalence of EHEC in meats:

It stands to reason that with the high prevalence of STEC in cattle, it is not surprising that meats are often contaminated with *E. coli* O157:H7 and non-O157 STEC. In a study of ground beef in Canada, less than 2% of the samples were positive for *E. coli* O157:H7 (Johnson et al. 1996), where as studies done by Acheson et al. (1996) and Samadpour et al. (1994) reported that none of the samples were positive for *E. coli* O157:H7. Doyle and Schoeni (1987) isolated *E. coli* O157:H7 in 3.4% of ground beef samples obtained from retail stores in Wisconsin and Alberta, Canada. The U.S. Department of Agriculture (USDA) as part of its National Microbiological Monitoring Program revealed that the prevalence of *E. coli* O157:H7 in ground beef products was less than 1% (Meng and Doyle 1998).

When compared to the prevalence of *E. coli* O157:H7, the prevalence of STEC in ground beef appears to be high. According to studies done by Clarke et al. (1994) in Canada, the prevalence of non-O157 STEC in ground beef ranges from 15 to 40%. Similar rates of prevalence have been reported for non-O157 STEC in the USA (23 to 25%; Acheson et al. 1996; Samadpour et al. 1994), Netherlands (16.1%; Heuvelink et al. 1996) and the United Kingdom (17%; Willshaw et al. 1993). The high prevalence of *E. coli* O157:H7 detected in cattle has not resulted in a high prevalence in ground beef. However, there is a high rate of non-O157:H7 STEC in ground beef from developed countries in North America and Europe. These finding suggest that more research must be done to find out how the ground beef is becoming contaminated with non-O157:H7 STEC.

2.6 EHEC and STEC infections in humans:

E. coli O157:H7 infections have been reported most frequently in Canada, the United States and the United Kingdom, although illnesses due to *E. coli* O157:H7 have been reported in over 30 countries on six continents (Doyle, 1991; Griffin and Tauxe, 1991; Waters et al., 1994; Boyce et al., 1995; Chapman, 1995; Mead and Griffin, 1998; Nataro and Kaper, 1998). *E. coli* O157:H7 is an important pathogen in Japan and Europe, whereas in countries such as Australia, Argentina, Chile and South Africa, non-O157 serotypes of EHEC are a more frequent cause of HC and HUS (Bettelheim, 1998; Nataro and Kaper, 1998). Mead and Griffin (1998) reported that the ratio of *E. coli* O157:H7 to non-O157:H7 STEC isolated from patients with diarrhea was 1.5:1.

Infections due to *E. coli* O157:H7 appear to be more common in Canada than in the United States (Griffin and Tauxe, 1991; Boyce et al., 1995). Within the United

States, outbreaks are apparently more common in the northern states than in the southern states (Griffin and Tauxe, 1991; Slutsker et al., 1997; Nataro and Kaper, 1998; Chapman, 2000). In Canada, reports of *E. coli* O157:H7 infections have been more frequent in the western provinces than in the east, with the exception of Prince Edward Island (Griffin and Tauxe, 1991; Nataro and Kaper, 1998). The highest annual provincial rates of STEC infection for the period between 1992 and 1995 occurred in Prince Edward Island (10.2 cases per 100,000 persons), Manitoba (7.6 per 100,000) and Alberta (7.4 per 100,000) (Spika et al., 1998).

In Canada, the incidence of HUS caused by non-O157 STEC is similar to that caused by *E. coli* O157:H7 (Spika et al., 1998; Arthur et al., 2002). Spika et al. (1998) reported that out of 31 cases of HUS, 12 were caused by *E. coli* O157:H7, where as 11 were caused by non-O157 STEC and eight were not caused by STEC. A study in Nebraska by Arthur et al. (2002) revealed that out of 14 cases of HUS, six were caused by *E. coli* O157:H7 and seven were caused by non-O157:H7 STEC. In Australia, 16% of HUS cases are due to *E. coli* O111:NM, 11% by *E. coli* O157:H7 and 14% are due to non-O157 STEC (Smith et al., 1998). The most common non-O157 STEC serotypes isolated in cases of diseases are O26, O111, O103, O113 and O128, and these serotypes are associated with 50% of STEC infections in Europe (Caprioli and Tozzi 1998, Nataro and Kaper, 1998). These results combined with the fact that only 3% of laboratories test for non-O157 STEC (Arthur et al., 2002) reveal that there is a need for increased monitoring of non-O157 STEC.

2.7 Methods for the detection of EHEC:

Many methods have been developed for the isolation and characterization of *E. coli* O157:H7; however, there are very few methods that can reliably be used to isolate non-O157 EHEC. Procedures traditionally used to detect faecal coliforms including *E. coli* use incubation temperatures ranging from 44 to 45°C (Prescott et al., 1996). However, studies by Doyle and Schoeni (1984) indicated that *E. coli* O157:H7 grows poorly at these temperatures. Methods developed to isolate and characterize EHEC combine the use of enrichment, differential and selective media along with immunological (immunomagnetic separation and ELISA) and genotypic methods (hybridizations, amplifications) (Vernozy-Rozand, 1997).

Most biochemical reactions for *E. coli* O157:H7 are typical of *E. coli*, with the exception of the inability to rapidly ferment sorbitol and the lack of β-glucuronidase activity (Vernozy-Rozand, 1997; Padhye and Doyle, 1992; Okrend et al., 1990a). Approximately 80 (Strockbine et al., 1998; Okrend et al., 1990a) to 93% (Vernozy-Rozand, 1997) of *E. coli* isolates ferment sorbitol in 24 h, where as 95% of *E. coli* O157:H7 strains do not. Similarly, 93 (Vernozy-Rozand, 1997) to 97% (Okrend et al., 1990a) of *E. coli* strains possess the enzyme β-glucuronidase, but the vast majority of *E. coli* O157:H7 strains do not. Using these characteristics differential media have been designed for the isolation of *E. coli* O157:H7 (Prescott et al., 1996). These media include sorbitol MacConkey agar (SMAC), on which sorbitol-fermenting colonies are pink, and sorbitol negative colonies are a white-gray colour (Farmer and Davies, 1985). Using this medium the small percentage (7 to 20%) of *E. coli* O157:H7 sorbitol-fermenting colonies would not be identified.

A second differential characteristic that can be used is the presence or absence of the β-glucuronidase enzyme. The substance, 4-methyllumbelliferyl-β-D glucuronide (MUG) can be added to a medium and produces a fluorescent product after cleavage by the β-glucuronidase enzyme (Padhye and Doyle, 1991). The drawback of this method is that the product of the reaction diffuses out of the colonies into the agar obscuring the colonial source of the enzyme. Two other compounds, 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (BCIG) and indoxyl-β-D-glucuronide (IBDG), can be supplemented into the media, giving a colour change after cleavage by the β-glucuronidase enzyme, so that positive colonies turn blue, and negative colonies remain white (Okrend et al., 1990a). Other differential media containing MUG are listed in Table 1. Differential media are good when working with pure cultures, but 10 to 20% of human gastrointestinal isolates do not ferment sorbitol, and at least 5% of *E. coli* O157:H7 isolates posses the β-glucuronidase enzyme (Strockbine et al., 1998). This has lead to the development of combinations of differential and selective media to improve the isolation of *E. coli* O157:H7.

Selective media can include antibiotics or other compounds that select for specific types of bacteria due to inherent or acquired resistance to these compounds. Selective media can combine selective and differential components to increase the effectiveness of the medium (Prescott et al., 1996). Examples of selective media for *E. coli* O157:H7 include cefixime-tellurite SMAC (CT-SMAC) (Zadik et al., 1993). Cefixime is added to the medium to suppress the growth of *Proteus* spp., which accounts for approximately 15% of non-sorbitol fermenting bacteria in faecal samples (Strockbine et al., 1998). Tellurite is added because the minimal inhibitory concentrations were higher for *E. coli*

Table 1: Selective and differential agar for the isolation of *E. coli* O157:H7.

Name	Composition (L^{-1})	Name
SMAC	MacConkey Agar D-Sorbitol, 1%	Farmer and Davis (1985)
CR-SMAC	MacConkey Sorbitol agar Rhamnose, 0.5% Cefixime, 0.05 mg	Chapman et al. (1991)
CT-SMAC	MacConkey Sorbitol agar Cefixime, 0.05 mg Potassium tellurite, 1mg	Zadik et al. (1993)
MSA-MUG	MacConkey Sorbitol agar MUG, 0.01%	Padhye and Doyle (1991)
MSA-BCIG	MacConkey Sorbitol agar 5-Bromo-4-chloro-3-indoxyl-β-D-glucuronic acid cyclohexylammonium salt, 0.01%	Okrend et al. (1990a)
PRS-MUG	Phenol red base +2% agar D-Sorbitol, 0.5% 4-Methyllumbelliferyl-β-D-glucuronide, 0.005%	Okrend et al. (1990b)
HC	Tryptone, 20 g Bile Salts #3 1.12 g Sodium chloride (NaCl), 5 g Sorbitol, 20 g MUG, 0.01% Bromocresol purple, 0.015 g	Szabo et al. (1986)
SD-39	Proteose peptone 5 g Yeast extract 3 g Sodium chloride 5 g L-Lysine monohydrochloride 10 g Dextrose 2.5 g, X-glucose 0.05 g D-Sorbitol 20 g Magnesium sulfate 7-hydrate 1.5 g Monensin 0.038 g, novobiocin 7.5 mg Sodium glucuronate 0.5 g Phenol red 0.12 g Agar 15 g	Entis and Lerner (1997)
EOH	Peptone 15.5 g Proteose peptone 3 g D-Sorbitol 10 g Bile salts #3 1.5 g NaCl 5 g, Agar 15 g Phenol red 0.036 g Indigo carmine 0.03 g	Kang and Fung (1999)

O157:H7 than for other *E. coli* and other non-sorbitol fermenting enterics including *Aeromonas* spp., *Plesiomonas* spp., and *Hafnia alvei* (Vernozy-Rozand, 1997; Zadik et al., 1993). BCIG-MacConkey sorbitol agar (BCIG-MSA) (Okrend et al., 1990b) and MSA-MUG (Strockbine et al., 1998: Padhye and Doyle, 1991) have also been developed to combine sorbitol fermentation and β-glucuronidase enzyme activity to improve isolation of *E. coli* O157:H7.

Escherichia coli O157:H7 is usually present in very low numbers in samples, thus direct plating has limited success when isolating this organism from food or environmental (faecal) samples (Vernozy-Rozand, 1997; Wallace and Jones, 1996). For this reason, culture media for enrichment have been developed to allow specific bacterial strains to multiply while the growth of competitive bacteria is inhibited. Most enrichment media for *E. coli* include bile salts, to inhibit the growth of gram-positive microflora (non-enterics), and antibiotics for increased selectivity. The most common enrichment broth medium is trypticase soy broth (TSB) supplemented with 1.5 g/L bile salts #3 and novobiocin (20 mg/L) or acriflavine (10 mg/L) to suppress growth of other gram-negative bacteria (Vernozy-Rozand, et al., 1997). Another common enrichment broth is buffered peptone water supplemented with vancomycin (8 mg/L), cefixime (0.05 mg/L) and cefsulodin (10 mg/L) (Chapman et al., 1994b). Vancomycin inhibits the growth of gram-positive bacteria, and cefsulodin and cefixime inhibit the growth of *Aeromonas* spp., and *Proteus* spp., respectively (Strockbine et al., 1998; Vernozy-Rozand, 1997; Chapman et al., 1994b). All enrichment media possess some form of buffering to keep the pH at an optimal level for bacterial growth. Formulas for other enrichment broth with similar characteristics required for increasing the number of *E. coli* O157:H7 relative to other microorganisms in samples as shown in Table 2.

Table 2: Enrichment broth media for isolation of EHEC.

Name	Composition (L^{-1})	Reference
mTSB	Trypticase soy broth, 30 g Bile salts #3, 1.5 g Dipotassium phosphate (K_2HPO_4), 1.5 g Novobiocin, 20 mg	Doyle and Schoeni 1987
dm TSB-CA	Trypticase soy broth, 30 g Bile salts #3, 1.5 g K_2HPO_4, 1.5 g Casamino acids, 10 g Acriflavine-HCL, 10 mg	Padhye and Doyle 1991
BPW-VCC	Buffered peptone water Vancomycin, 8 mg Cefixime, 0.05 mg Cefsulodin, 10 mg	Chapman et al. 1994b
mEC-n	Tryptone, 20 g Bile salts #3, 1.12 g Lactose, 5g K_2HPO_4, 4 g KH_2PO_4 1.5 g NaCl, 5 g Novobiocin, 20 mg	Organon Teknica 1993
sTSB	sTSB medium (TSB mondified with TCA cycle intermediates, not specified) Bile salts #3 1.5 g Oxyrase™ (amount not specified) Phosphate buffer (type not specified) Novobiocin 20 mg	Chapman et al. 2001

After growth in enrichment media, a concentration or separation method can be used to isolate *E. coli* O157:H7, and limit the interference from food debris and background microorganisms, thus increasing sensitivity (Vernozy-Rozand, 1997; Hindle et al., 1995; Sanderson et al., 1995; Zhao et al., 1995). Many techniques have been studied for this purpose, including centrifugation (Basel et al., 1983), filtration (Bobbitt et al., 1993), lectin-based biosorbents (Payne et al., 1992), aqueous biphasic systems

(Bennet et al., 1994), ultrasound (Miles et al., 1995), and IMS (Chapman et al., 2001; Wallace and Jones, 1996; Wright et al., 1994). Centrifugation uses a low speed centrifugation to separate large debris from the cell suspensions (Basel et al., 1983). Filtration achieves the same result by filtering out large debris while leaving the bacterial cells in suspension (Bobbitt et al., 1993). All of these methods concentrate bacterial cells, but not specific strains. Immunomagnetic separation allows the concentration and separation of target cells from the background microflora. IMS reduces the total analysis time and improves sensitivity of detection. Para-magnetic particles are coated with antibodies specific to target organisms (de Boer and Beumer, 1999; Strockbine et al., 1998; Vernozy-Rozand, 1997; Wallace and Jones, 1996). There have been many studies that use beads coated with anti-O157 (Chapman and Siddons, 1996; Cubbon et al., 1996; Karch et al., 1996), anti-O26 or anti-O111 antibodies (Safarikova and Safarik, 2001), targeted to the O-antigen of these specific strains of *E. coli*. The target organism is captured onto the magnetic particles and the whole complex is removed from the system by application of a magnetic field. Target organisms are removed from food debris and background microorganisms, and by resuspending isolated cells in a reduced volume, concentration can be achieved (Wallace and Jones, 1996). Using IMS and spreading beads onto one of the selective media shown in Table 1, this method is one day quicker and 100 to 1000 times more sensitive than isolation of *E. coli* O157:H7 by other non-IMS methods (Wright et al., 1994). The most sensitive IMS method was reported by Bolton et al. (1996), which uses mTSB-n as an enrichment broth followed by spread plating IMS beads onto CT-SMAC.

The majority of media used in the isolation of EHEC focus on *E. coli* O157:H7, O26 and O111. Isolation of non-O157 EHEC has been done using the lack of rhamnose fermentation specific to *E. coli* O26 by plating onto RMAC or CT-RMAC (Hiramatsue et al., 2002) (Table 3). This is similar to CT-SMAC, which is used for *E. coli* O157:H7 but uses the lack of rhamnose fermentation instead of sorbitol. This medium works well for the isolation of *E. coli* O26 but not many other non-O157:H7 EHEC (Hiramatsue et al., 2002). Another medium developed by Beutin et al. (1988) uses the production of an accessory virulence factor enterohaemolysin (EhxA) by most EHEC and STEC strains as a differentiating characteristic. This medium is composed of tryptose blood agar, supplemented with 5% washed sheep blood and $CaCl_2$ (WBA). Enterohaemolysin produces small turbid zones of inhibition different from the standard α-haemolysis (Beutin et al., 1988). WBA is able to identify 74 (Beutin et al., 1988) to 76% (Sugiyama et al., 2001) of EHEC strains. Lehmacher et al. (1998), increased the selectivity of the medium by adding selective antibiotics (VCCWBA; Table 3) that prevent growth of background microflora and increase the secretion of enterohaemolysin into the agar. Using this medium 83 (Sugiyama et al., 2001) to 88.4% (Lehmacher et al., 1998) of EHEC isolates were identified. The addition of mitomycin to the original WBA (WBMA) by Sugiyama et al. (2001) increased the isolation to 97%, but this compound is extremely mutagenic and undesirable to work with in the laboratory. A reliable medium for the isolation of all EHEC strains has yet to be developed.

Table 3: Solid medium for isolation of non-O157:H7 EHEC based on production of enterohaemolysin.

Name	Composition (L^{-1})	Reference
WBA	Tryptose blood agar 40 g Defibrinated washed sheep blood in Phosphate buffered saline (PBS) 50 mL Calcium chloride ($CaCl_2$) 441 mg	Beutin et al. 1988
VCCWBA	Tryptose blood agar base 33 g Tryptose 5 g Soluble starch 5 g $CaCl_2$ 441 mg Agar 3 g Difibrinated washed sheep blood in PBS 30 mL Vancomycin 30 mg Cefixime 0.02 mg Cefsulodin 3 mg	Lehmacher et al. 1998
WBMA	Tryptose blood agar 33 g Tryptose 5 g Defibrinated washed sheep blood in PBS 50 mL $CaCl_2$ 441 mg Mitomycin C 5 mg	Sugiyama et al. 2001
RMAC	MacConkey agar base no lactose 40 g Rhamnose 10 g	Hiramatsu et al. 2002
CT-RMAC	MacConkey agar base no lactose 40 g Rhamnose 10 g Tellurite 2.5 mg Cefixime 0.05 mg	Hiramatsu et al. 2002

Immunochemical assays should prove to be a significant improvement over standard culture methods for the detection of foodborne pathogens (Meng et al., 2001). These techniques take advantage of the specificity and sensitivity of the antibody (Ab) and antigen (Ag) reaction (similar to IMS) for detection (de Boer and Beumer, 1999). These techniques include immunochromatography, immunoprecipitation and ELISA. Immunoprecipitation reactions are based on the same technology as the home pregnancy tests, where there is a quick precipitation reaction if the antibody binds the antigen. All the immunoprecipitation methods for isolation of EHEC were developed based on the detection of the O157 antigen. This only allows identification of *E. coli* O157, and does not detect the H7 antigen (Meng et al., 2001; Strockbrine et al., 1998; Chapman et al., 1997b). ELISA is a useful form of an immunochemical method for the detection of foodborne pathogens in the food-processing setting, based on their simplicity and the ability of laboratories to analyze large numbers of samples at a time (Meng et al., 2001; Vernozy-Rozand, 1997). Most ELISAs are typical sandwich assays where the capture antibody is immobilized on a solid support (microtitre plate). The test sample is added and the sample is incubated. The unbound sample is removed by washing and a reporter antibody conjugate (usually an enzyme like alkaline phosphatase) is added. After another incubation period the unbound reporter conjugate is washed and the reporter substrate is added and a colour change takes place if the reporter antibody is present (de Boer and Beumer, 1999). Most ELISAs are also geared towards the O157 antigen with a few tests also targeting the H7 antigen (Grif et al., 1998; Chapman et al., 1997b; Vernozy-Rozand, 1997; Padhye and Doyle, 1991). The EHEC-TEK® ELISA identifies *E. coli* O157:H7 and *E. coli* O26:H11 (Meng et al., 2001).

Enzyme linked immuno-sorbent assays have also been developed to detect the presence of shiga-like toxins in broth medium, but most tests cannot differentiate between Stx-1 and Stx-2 (Atalla et al., 2000; Law et al., 1994; Acheson et al., 1990). These assays are able to identify any Shiga toxin producing strain that may be present, not just EHEC. Concerns with immunochemical techniques include problems with cross-reactivity and difficulties obtaining species specific assays. Also, most immunochemical methods continue to require an enrichment technique; however, subsequent identification is quite rapid when compared with standard culture methods (Meng et al., 2001;

Strockbine et al., 1998; Vernozy-Rozand, 1997). A partial list of available immunological tests for isolation of *E. coli* O157:H7 and Stxs are shown in Table 4.

Table 4: List of commercially available assays for detection of EHEC serotypes and shiga toxins in foods[a].

Target	Trade Name	Assay Format[b]
O157 Antigen	PetrifilmHEC	Blot-ELISA
	EZ COLI	Tube-ELISA
	Assurance	ELISA
	TECRA	ELISA
	VIP	Ab-ppt
	Reveal	Ab-ppt
	Now	Ab-ppt
	QUIX	Ab-ppt
	VIDAS	ELISA
O157:H7 & O26:H11	EHEC-TEK	ELISA
Shiga toxins 1 & 2	VEROTEST	ELISA
	Premier EHEC	ELISA
	Verotox-F	RPLA
	VTEC	RPLA

[a] Table modified from: Meng et al. (2001).
[b] ELISA, enzyme-linked immuno-sorbent assay; ab-ppt, immunoprecipitation or immunochromatography, RPLA, reverse passive latex agglutination.

Genotypic methods are the third category of techniques that can be used to identify EHEC. These methods can be divided into two categories; virulence testing and typing or sub-typing of microorganisms. Virulence testing is usually done with polymerase chain reaction (PCR) or DNA-DNA hybridizations (colony hybridization, slot-blot hybridization or reverse dot-blot hybridization). Typing or sub-typing methods can be done using pulsed-field gel electrophoresis (PFGE), DNA sequencing or PCR based methods that include: random amplification of polymorphic DNA (RAPD) or PCR-restriction fragment length polymorphism (PCR-RFLP) (Meng et al., 2001; de Boer and Beumer, 1999; Strockbine et al., 1998; Vernozy-Rozand, 1997).

Identification of EHEC can be done without the isolation and purification of colonies using gene probes developed to identify specific virulence genes. Virulence testing is based on genotypic characteristics of the pathogen (Padola et al., 2002; Strockbine et al., 1998; Vernozy-Rozand, 1997). There are numerous DNA probes that have been developed for the detection of EHEC virulence genes, the most common of these are targeted to the Shiga-toxin genes (*stx*-1 and *stx*-2), the *E. coli* attachment and effacement (*eaeA*) genes and enterohaemolysin (*ehxA*) gene (Paton and Paton, 2002; Call et al., 2001; Kobayashi et al., 2001; Radu et al., 2001; Pass et al., 2000; Fagan et al., 1999; Meng et al., 1998; Wang et al., 1997; Cebula et al., 1995; Gannon et al., 1993; Bettelheim et al., 1990). There is also a PCR developed to detect the unique base mutation in the *uidA* gene of *E. coli* O157:H7, which would indicate an increased chance of pathogenicity (Feng, 1993). These gene probes can be used in colony hybridizations or slot-blot or dot-blot hybridizations. These methods are time consuming and labour intensive (de Boer and Beumer, 1999; Vernozy-Rozand, 1997). A reverse dot-blot microarray hybridization procedure has been developed where the probes are immobilized on a solid surface (usually a nylon membrane) and the sample is prepared doing a multiplex PCR reaction and then probed for all virulence factors at one time. This method is less time consuming and allows the identification of multiple genes at one time. Although it involves the use of either radioactive material or fluorescence, which can lead to problems in interpretation of results, PCR can be used as an alternative to DNA hybridization to identify virulence genes. Polymerase chain reaction is rapid and cost effective for the majority of laboratories, although in most cases samples must be purified to prevent inhibition of reactions (Strockbine et al., 1998). The high sensitivity can be a problem because the mint amount of contamination can cause false positive results (Johnson, 2000). Polymerase chain reaction amplifies virulence genes, but does not permit the isolation or identification of specific strains of EHEC.

The ultimate method for identification of strains of EHEC would be DNA sequence analysis to identify what the bacterium is and which genes it posses. However, this method is very time consuming, technologically demanding, not cost effective for most laboratories and also requires isolation of cultures. There is also the possibility that the strain has not been sequenced before and therefore a reference strain to compare the sequence to would not be available (Olive and Bean, 1999; Strockbine et al., 1998). For this reason DNA subtyping methods are commonly used to prove that strains are related, a list of the advantages and disadvantages of DNA subtyping methods are listed in Table 5. The gold standard for subtyping is total genomic DNA, PFGE, an electrophoretic method that utilizes an infrequent cutting restriction enzyme to cut the genome into various molecular length DNA fragments that range from 10 to 800 kb. The digest is then run in an agarose gel that is exposed to an electrical current that can change direction as desired, which allows clear separation of the DNA fragments. This method is highly discriminatory and superior to most methods of analysis of *E. coli*. This method is time consuming (2 to 3 d) and can reduce the ability to analyze large numbers of samples (de Boer and Beumer, 1999; Olive and Bean, 1999).

Polymerase chain reaction-restriction fragment length polymorphism analysis utilizes polymorphisms in specific genes to identify similarities or differences of microorganisms. A combination of PCR amplification and restriction digestion is used to allow subtyping of a microorganism. A specific locus to be examined, such as the

Table 5: DNA-based typing methods for microorganisms[a].

Methodology[b]	Ease of use	Ease of interpretation	Discrimination power	Time to results (d)	Reproducibility
PFGE	Moderate	Easy	High	3	Good
PCR-RFLP	Easy	Easy	Moderate	1	Good
RAPD	Easy	Easy	High	1	Moderate
Rep-PCR	Easy	Easy	High	1	Good
CFLP	Moderate	Moderate	Moderate	2	Good
AFLP	Moderate	Easy	High	2	Good
Sequencing	Difficult	Moderate	High	2	Good

[a] Modified from Olive and Bean (1999).

[b] PFGE, Pulsed-Field gel electrophoresis; PCR-RFLP, Polymerase chain reaction restriction fragment length polymorphism; RAPD, Randomly amplified polymorphic DNA; Rep-PCR, Repetitive extragenic palindromic polymerase chain reaction; CFLP, Cleavage fragment length polymorphism; AFLP, Amplified fragment length polymorphism.

flagellar antigen, is amplified with gene specific primers and digested with a restriction enzyme. Fragments of DNA are separated on an agarose gel and the digestion pattern is analyzed. The polymorphisms of different organisms will give different banding patterns (Olive and Bean, 1999; Strockbine et al., 1998). This method has been used to analyze the flagellar antigen (*fliC* gene) of *E. coli* (Johnson and Stell, 2001; Wang et al., 2000; Fields et al., 1997). This method is easy to use and analyze; however, the discriminatory power is lower than that of PFGE.

Randomly amplified polymorphic DNA assays are based on the use of short random sequence primers (9 to 10 bases in length) that hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures. This allows the primers to bind and initiate amplification of regions in the bacterial genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the primers is produced. The number of these primer sites and lengths vary among different bacterial strains or species. Separation of the amplification products by agarose gel electrophoresis reveals a pattern of bands, which in theory is characteristic of the particular bacterial strain. This method is more discriminatory than PCR-RFLP but lacks reproducibility and standardization. Many of the priming events are the result of imperfect hybridizations between the primer and target site (Franklin et al., 1999; Olive and Bean, 1999; Strockbine et al, 1998), and amplification is extremely sensitive to changes in the annealing temperature, which can lead to variability in banding patterns (Franklin et al., 1999). Randomly amplified polymorphic DNA has been used most often to characterize *E. coli* O157:H7 strains (Birch et al., 1996; Madico et al, 1995).

Although the presence of *Escherichia coli* O157:H7 is of significant concern for the safety of the meat supply, there are many other strains of non-O157:H7 STEC that can cause the same illnesses as *E. coli* O157:H7. These strains do not have one specific trait that allows for their isolation from complex samples. The production of EhxA is one phenotypical trait that allows for the isolation of the majority of EHEC strains from food and bovine samples. The low numbers of EHEC in relation to the background microflora has required the development of sensitive genotypic methods for the characterization of EHEC and STEC strains in food and bovine samples. Many DNA typing methods are available that allow the comparison of the DNA from EHEC or STEC to compare their relatedness.

2.8 Objectives of research project:

The overall objective of this research was to evaluate the use of different methods to determine the prevalence of potentially pathogenic strains of *E. coli* at different stages of cattle production and processing and in the resulting ground beef. The first objective was to characterize pathogenic strains of *E. coli,* collectively known as EHEC, that were isolated from samples obtained at different stages of the beef production continuum. This included faecal samples obtained while cattle were in the pasture or feedlot, and hide, caecal and ground beef samples that were obtained during slaughter and processing. The second objective was to use PCR to determine the presence of *ehxA, eaeA, stx*-1, and *stx*-2 virulence genes in *E. coli* that were present in the samples obtained at different times during cattle production and processing.

3 Materials and Methods

3.1 Bacterial strains:

Strains used in this study are listed in Table 6. All strains of *E. coli* were stored at –70°C in Tryptic Soy broth (Becton, Dickinson and Co. (BD), Sparks, MD) modified with 0.15% bile salts #3 (Sigma-Aldrich Canada Ltd., Oakville, ON) and 0.15% dipotassium hydrogen phosphate (BDH Laboratory Supplies, Mississauga ON) (mTSB) supplemented with 12.5% glycerol (v/v) (Co-op, Lacombe, AB). *Salmonella, Yersinia,* and *Streptococcus* spp. were stored at –70°C in Tryptic Soy broth (TSB) supplemented with 12.5% glycerol (v/v). *Listeria monocytogenes* and *L. innocua* were stored at –70°C in All Purpose Tween (APT; BD) broth adjusted to pH 6.5 and supplemented with 12.5% glycerol (v/v). Cultures to be used in experimental procedures were obtained by inoculating frozen cells into the appropriate broth medium and incubating at 37°C for 18 to 20 h. Transfer was done twice before cultures were used in an experiment.

3.2 Animals and slaughter:

Hereford x Angus cross cattle (15 heifers and 15 steers) were placed on pasture after they were weaned (May 2000). After 138 d on pasture, cattle were transferred to a feedlot (October 2000), with the 15 heifers in one pen and 15 steers in an adjacent pen, and held for 118 d. While on feedlot, the cattle were fed a ration of barley silage and barley grain supplemented with a feedlot ration. Once a month during pasture grazing and feedlot finishing cattle were moved to a handling facility to collect routine performance data and faecal samples (described below). Cattle were slaughtered in two groups of 15 (heifers and steers on separate days) on March 21st and April 3rd 2001

Table 6: Bacterial strains used in this study.

Organism	Reference
Escherichia coli ATCC 25922	ATCC[a]
E. coli GGG 3 to 10 (inclusive)	GGG[b]
E. coli O157:H7 ATCC 43895	ATCC
E. coli O8:?	AAFC-PRC
E. coli O103:H2	AAFC-PRC
E. coli O153:H25	AAFC-PRC
E. coli O163:NM	AAFC-PRC
E. coli O5:NM	AAFC-PRC
E. coli O26:H11	AAFC-PRC
E. coli O111:NM	AAFC-PRC
E. coli O117:NM	AAFC-PRC
E. coli O120:H5	AAFC-PRC
E. coli O157:H7 (40)[d]	AAFC-PRC
E. coli O157:H7 (54)[d]	AAFC-PRC
E. coli O157:H7 (179)[d]	AAFC-PRC
E. coli O157:H7 (181)[d]	AAFC-PRC
E. coli O157:H7 (187)[d]	AAFC-PRC
E. coli O157:H7 (188)[d]	AAFC-PRC
Streptococcus bovis ATCC 15351	ATCC
Yersinia enterocolitica ATCC 23715	ATCC
Salmonella enterica serovar Typhimurium ATCC 13311	ATCC
Listeria monocytogenes ATCC 15313	ATCC
L. innocua ATCC 33090	ATCC
Hafnia alvei ATCC 13337	ATCC

[a] ATCC = American Type Culture Collection,

[b] GGG = G. G. Greer (Agriculture and Agri-Food Canada (AAFC) Lacombe Research Centre, AB,Canada),

[c] AAFC-PRC = AAFC Pacific Agri-Food Research Centre laboratory culture collection (Summerland, BC),

[d] Number used at SBC to differentiate *E. coli* O157:H7 strains.

at a federally inspected research abattoir. At the time of slaughter animals were approximately 13 months old and had an average weight of 564 kg.

3.3 Collection of samples:

A summary of experimental procedures done in this study is provided in Figure 3. Faecal grab samples were collected monthly from June 2000 until March 2001, while the cattle were on pasture and in the feedlot. Approximately 50 g of faecal material per animal was aseptically collected and placed into a sterile 4 oz polypropylene specimen container (Fisher Scientific Ltd., Nepean, ON). All faecal samples were frozen at -20°C until further processing. To obtain samples from the hide, a 500 cm^2 area of the rump was vigorously rubbed with a sterile Nasco Speci-Sponge (VWR Canlab, Mississauga, ON). This was repeated on the brisket with a separate sponge. Caecal samples were collected aseptically at slaughter by isolating the caecum, making a small incision and collecting approximately 50 g of caecal material into a sterile 4 oz polypropylene specimen container. Caecal and hide samples were held on ice for no more than 4 h before processing.

Ground beef with 20% fat was prepared from individual animals using trim from the rump and brisket areas. Meat was ground using a TS-12 meat grinder (Russell Food Equipment Ltd., Edmonton, AB). Meat from each animal was processed separately by grinding the meat through a 10 mm plate and then through a 5 mm plate. Approximately 500 g of ground beef was collected at the mid point of grinding for microbiological analysis. Approximately 300 g of ground beef per animal was immediately vacuum packaged and frozen (–20°C) until further processing. After meat from each sample was

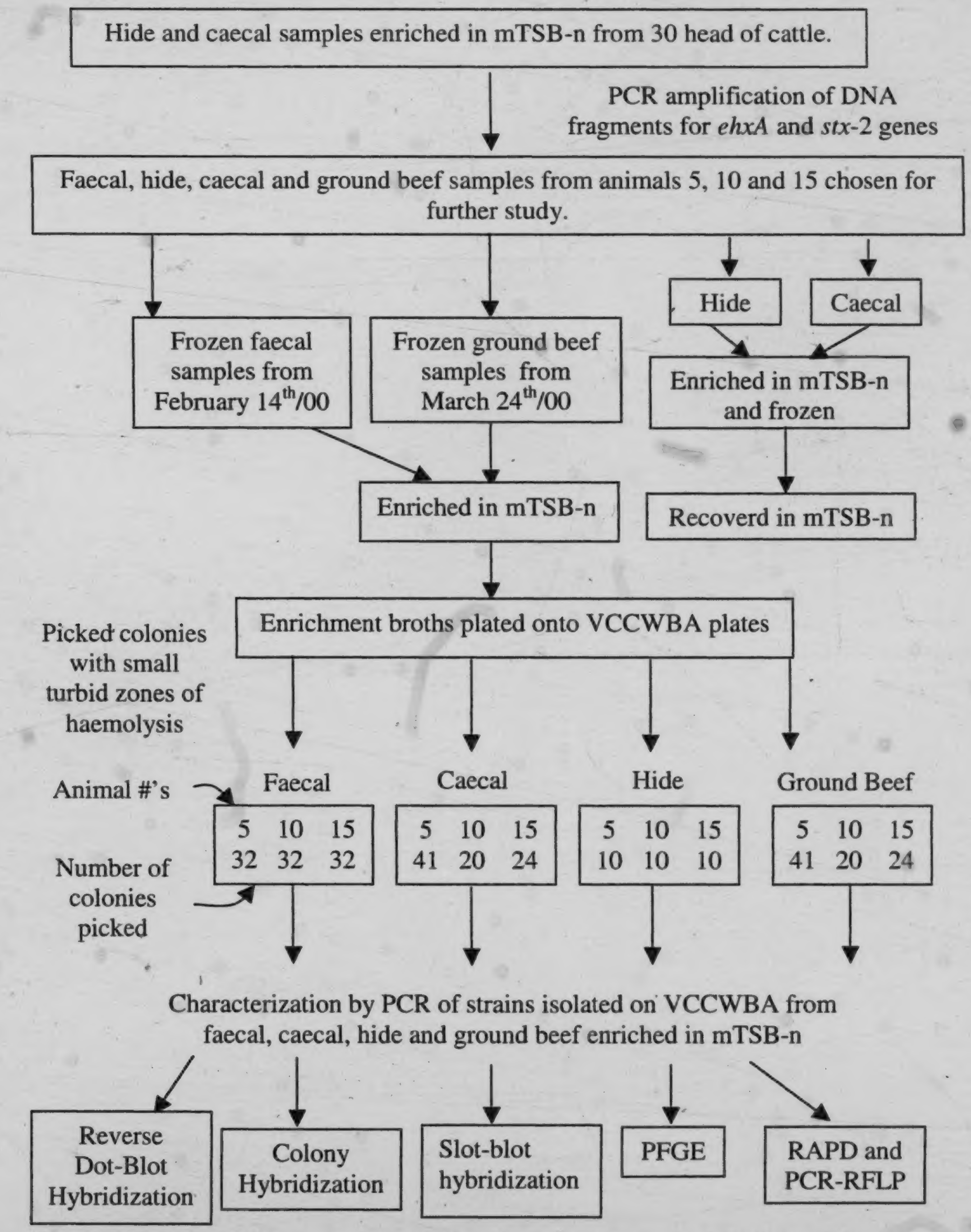

Hide and caecal samples enriched in mTSB-n from 30 head of cattle.
PCR amplification of DNA fragments for ehxA and stx-2 genes
Faecal, hide, caecal and ground beef samples from animals 5, 10 and 15 chosen for further study.
Hide
Caecal
Frozen faecal samples from February 14th/00
Frozen ground beef samples from March 24th/00
Enriched in mTSB-n and frozen
Enriched in mTSB-n
Recoverd in mTSB-n
Enrichment broths plated onto VCCWBA plates
Picked colonies with small turbid zones of haemolysis
Animal #'s
Number of colonies picked
Faecal
5 10 15
32 32 32
Caecal
5 10 15
41 20 24
Hide
5 10 15
10 10 10
Ground Beef
5 10 15
41 20 24
Characterization by PCR of strains isolated on VCCWBA from faecal, caecal, hide and ground beef enriched in mTSB-n
Reverse Dot-Blot Hybridization
Colony Hybridization
Slot-blot hybridization
PFGE
RAPD and PCR-RFLP

Figure 3: Schematic diagram illustrating the procedures used in the isolation and characterization of *E. coli*. mTSB-n, modified tryptic soy broth supplemented with novobiocin. PFGE, Pulsed field gel electrophoresis. VCCWBA, Vancomycin cefixime, cefsulodin washed sheep blood agar. RAPD, Randomly amplified polymorphic DNA. PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism.

ground, removable parts from the grinder were disassembled and rinsed with 85°C pressurized water to clean the grinder and reduce cross-contamination.

3.4 Preparation of antibiotics and media supplements:

Antibiotics were prepared as aqueous solutions using sterile milli-Q water (mQ H_2O) as follows: vancomycin (Sigma-Aldrich) 25 mg/mL, cefsulodin (Sigma-Aldrich) 30 mg/mL, cefixime (Dynal®Inc., Lake Success, NY) 1 mg/mL, novobiocin (Sigma) 5 mg/mL and tellurite (Dynal®) 50 mg/mL. After each antibiotic was completely dissolved solutions were filter sterilized using a 0.20 µm filter (VWR) syringe apparatus.

3.5 DNA isolation:

Isolation of DNA was done using two techniques. The first method of isolation was done by suspending colonies in 100 µL of mQ H_2O, or by centrifuging 1.5 mL of enrichment broth in an Eppendorf centrifuge 5415C (Brinkman Instruments Inc., Mississauga, ON) at 13,000 x *g* for 2 min. The pellet was resuspended in 1.5 mL of 0.9% w/v saline. Samples were centrifuged (Eppendorf 5415C) at 13,000 x *g* for 2 min and the pellet was resuspended in 100 µL of mQ H_2O. DNA samples were placed in a boiling water bath for 15 min to lyse cells and the DNA was then frozen in 1.5 mL eppendorf tubes at –20°C until required.

Alternately, isolation of DNA from enrichments samples was done using Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI). Two 1.5 mL portions of each enrichment broth were centrifuged in an Eppendorf 5415C centrifuge at 13,000 x *g* for 2 min. The supernatant was removed from each tube and 600 µL of nuclei lysis solution was added to one of the tubes and mixed by gentle pipetting. The lysate was transferred to the second tube and that pellet was lysed by gentle mixing. The lysate was incubated at 80ºC for 5 min to ensure complete lysis of the cells. After heating, the solution was allowed to cool at room temperature for 5 min (after each incubation from this point forward samples were allowed to return to room temperature). After cells were lysed, 3 µL of RNase solution [RNase A 4 mg/mL in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8.0)] was added to the cell lysate and the tubes were inverted 5 times to mix thoroughly. The samples were incubated at 37°C for 1 h to ensure complete removal of RNA. After the samples equilibrated to room temperature, 200 µL of protein precipitation solution was added to the RNase-treated cell lysate. The samples were vortexed vigorously for 20 s and then incubated on ice for 5 min to precipitate all proteins. Samples were centrifuged (Eppendorf 5415C) at 13,000 x *g* for 3 min and the supernatant containing the DNA was transferred to a 1.5 mL microcentrifuge tube containing 600 µL of room temperature isopropanol (BDH). Samples were mixed by inversion until strands of DNA formed a visible mass in the microcentrifuge tube. Samples were centrifuged (Eppendorff 5415C) at 13,000 x *g* for 2 min to pellet the DNA, and the supernatants were decanted. Tubes were inverted on clean absorbent paper for 1 min to ensure all isopropanol had been removed from the tubes. After complete evaporation, 600 µL of room temperature 70% ethanol (Fischer Scientific) was added to each tube and each pellet was washed by inversion and centrifuged at 13,000 x *g* for 2 min. The supernatant was then gently aspirated and the tubes were inverted on clean absorbent paper to air dry for 15 min. DNA was re-hydrated using DNA rehydration solution [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)] and incubated at 65°C for 1 h, inverting every 10 min. After incubation, 1 µL of each sample was visualized in a 1%

agarose gel, to ensure that genomic DNA had been isolated. All samples were stored at 4°C until further processing.

3.6 Enrichment of faecal, hide, caecal and ground beef samples:

Faecal, hide, caecal and ground beef samples from 30 head of cattle were enriched in mTSB supplemented with novobiocin (m-TSB-n) to help eliminate competitive microflora, while increasing the number of *E. coli* in each sample. Faecal samples were thawed at 4°C for 24 to 30 h and then approximately 25 g of faecal material was added to 225 mL of mTSB-n.

Caecal samples were enriched by weighing approximately 35 g of caecal material into a sterile medical stomacher 400 filter bag (Seward, London, UK), with 90 mL sterile 0.1% w/v peptone water (BD) and mixing with a Seward 400 mark 2 stomacher (Seward) on low speed. After mixing, all liquid was poured into sterile 250 mL screw cap polypropylene centrifuge bottles (Mandel Scientific Ltd., Guelph, ON) and centrifuged, in a Sorvall® RC-5B refrigerated Superspeed centrifuge at 1000 x *g* for 10 min to remove large faecal debris from the sample. This centrifugation step was done to aid in the elimination of PCR inhibitors. The supernatant was decanted into a sterile centrifuge bottle and centrifuged (Sorvall® RC-5B) at 16,000 x *g* for 10 min to pellet the cells. The supernatant was removed and the pellet was resuspended in 0.15 times the original volume of 0.1% peptone water (0.15 w/v). For the final enrichment, 2 mL of the cell suspension was added to 225 mL of mTSB-n.

Samples taken from the hides were prepared as above, except that each sponge (rump and brisket) was mixed in 30 mL of 0.1% w/v peptone water and after mixing the liquid from both samples were combined. After centrifugation the pellet was resuspended in 8 mL of 0.1% w/v peptone water.

Ground beef samples were thawed at 4°C for 24 to 30 h and then approximately 25 g of ground beef was added to 225 mL of mTSB-n. After preparation, all enrichment broths were incubated at 35°C for 20 to 24 h and then samples were prepared as needed (DNA isolation or plating on media for isolation). A 1.5 mL portion of each enrichment was stored at –70°C in 12.5% (v/v) glycerol (in triplicate).

3.7 Polymerase chain reaction:

Polymerase chain reaction was used to amplify portions of DNA, which encode virulence genes for EHEC. The DNA sequence of the primers used to test for the presence of virulence genes and the *E. coli* markers *uidA* and *uspA* by PCR were taken from the literature [*Stx*-1, *Stx*-2 and *uidA* (Cebula *et al.* 1995), *eaeA* (Kobayashi *et al.* 2001), *uspA* (Chen and Griffiths, 1998) and *ehxA* (Wang *et al.* 1997)] and the primers were synthesized by Sigma-Aldrich. Each PCR reaction had to be optimized to find the best annealing and amplification conditions. Optimization of PCR conditions included determination of the appropriate concentrations of $MgCl_2$, dATP, dGTP, dTTP, dCTP, template and the appropriate temperature. Temperature of annealment was assumed to be near the melting temperature of the primers (T_m). Each primer was tested from 5°C below to 5°C above the T_m, at approximately 1°C intervals. At each temperature, gradients of $MgCl_2$ (Sigma) ranging from 1.5 mM to 4.5 mM were tested for the best banding results. To determine which concentrations of dNTPs (Life Technologies Burlington, ON) provided the brightest banding, dNTPs were tested at concentrations ranging from 0.125 mM to 0.1 mM. Template concentrations were tested depending on whether they were crude isolates or purified DNA to test for inhibition of PCR. PCR products were compared using appropriate positive and negative controls for every reaction.

After optimization of primers, all PCR amplifications were carried out in 25 µL volumes in 0.2 mL thin walled striptube and cap PCR tubes (VWR). PCR master mixes contained 1X PCR buffer [50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3](Sigma), with a total of 3.0 mM $MgCl_2$. For the *eaeA* primer a concentration of 3.5 mM $MgCl_2$, 0.25 mM dNTPs, 50 pmol of each primer (Sigma), 1 unit of *Taq* DNA Polymerase (Sigma) was required. Amplification was done using a Robocycler Infinity thermocycler with a hot top (Stratagene Cloning Systems, La Jolla, CA) with one cycle at 94°C for 2 min and 35 cycles of 94°C with annealing and extension as described in Table 7, and a final extension of 72°C for 5 min.

Amplification products were analyzed using submarine gel electrophoresis (5 volts/cm between electrodes) in 1.5% agarose gels (Sigma-Aldrich). All gels were run with a 100 bp (0.5 µg) DNA molecular size marker (Life Technologies) and the gels were stained in a water solution containing 12 mg/L of ethidium bromide (Sigma-Aldrich) for 1 h. Gels were viewed under UV light using the Kodak DC 290 zoom digital camera in combination with the Electrophoresis Documentation and Analysis System (EDAS) 290 and 1D image analysis software version 3.5.2 (Eastman-Kodak, Rochester NY).

3.8 Sequencing of PCR products:

To ensure that the proper targets were being amplified during PCR, sequence analysis for *ehxA*, *stx*-1, *stx*-2, *eaeA*, *uspA*, and *uidA* gene fragments was performed. PCR amplification of DNA fragments was from purified genomic DNA isolated from *E.*

Table 7: Primers sequences, time and temperature of annealment used to amplify specific fragments of DNA for *stx*-1, *stx*-2, *eaeA*, *ehxA*, *uidA* and *uspA* genes.

Target	Primer Name	Size (bp)[a]	Sequence	Temp & Time
stx-1	LP30	348	5'-CAGTTAATGTGGTGGCGAAGG– 3'	65°C 90s[a]
	LP31		5'–CACCAGACAATGTAACCGCTG–3'	72°C 90s[b]
stx-2	LP43	584	5'-ATCCTATTCCCGGGAGTTTACG–3'	65°C 90s[a]
	LP44		5'-GCGTCATCGTATACACAGGAGC-3'	72°C 90s[b]
eaeA	EaeA1	815	5'-ACGTTGCAGCATGGGTAACTC–3'	60°C 60s[a]
	EaeA2		5'-GATCGGCAACAGTTTCACCTG–3'	72°C 60s[b]
ehxA	EHEC-3	361	5'-GTAGGGAAGCGAACAGAG–3'	65°C 45s[a]
	EHEC-4		5'-AAGCTCCGTGTGCCTGAA–3'	72°C 45s[b]
uidA	PT-2	252	5'-GCGAAAACTGTGGAATTGGG-3'	65°C 90s[a]
	PT-3		5'-TGATGCTCCATAACTTCCTG-3'	72°C 90s[b]
uspA	UspA F	884	5'-CCGATACGCTGCCAATCAGT–3'	70°C 60s[a]
	UspA R		5'-ACGCAGACCGTAGGCCAGAT–3'	72°C 60s[b]

[a] Length in base pairs
[b] Amplification time and temperature
[c] Extension time and temperature

coli O157:H7 (ATCC 43895), *E. coli* O157:H7 (SBC 187) and *E. coli* O157:H7 (SBC 188). Following amplification 80 µL of each PCR product was purified by a mini- Quick Spin DNA Column (Roche Diagnostic Canada, Laval, QC) using centrifugation (Eppendorf 5415C) at 1000 x *g* for 4 min, to ensure purity of the amplified product. After purification, 10 µL of the product was run on a 1.5% agarose gel to certify that the product had been properly purified and the correct product had been amplified according to its published size (Table 7). Nucleotide sequences were determined on both strands with the cycle sequencing method using the DYEnamic ET sequencing kit (Amersham Pharmacia Biotech, Baie d'Urfe, QC) on an automated ABI 377 DNA sequencing machine (Perkin-Elmer Applied Biosytems, Foster City, CA). Sequences were aligned with sequences in the GenBank database using the BLAST algorithms for local alignments (Altschul et al., 1990).

3.9 Initial screening of enrichment broths for the *ehxA* and *stx*-2 genes using PCR:

Enrichment broths prepared from hide and caecal samples from all 30 animals were analyzed by PCR amplification for the presence of DNA from the *ehxA* and *stx*-2 genes. This was done to determine which enrichment broths would be used for further analysis. After hide and caecal samples, had been enriched, 1mL of enrichment broth was placed in a 1.5 mL Eppendorf microcentrifuge tube and centrifuged in an Eppendorf 5415C centrifuge at 13,000 x *g* for 5 min to pellet the cells. The supernatant was removed and the cells were washed in 1 mL of sterile 0.9% w/v saline solution and centrifuged (Eppendorf 5415C) at 13,000 x *g* for an additional 5 min to repellet cells. This step was repeated twice. After the final centrifugation, cells were resuspended in 0.5 mL of sterile H_2O. Samples were placed in a boiling water bath for 15 min to lyse the cells and then frozen until PCR was done. Based on these results animals 5, 10 and 15 were chosen for further isolation and characterization of EHEC strains using methods outlined below.

3.10 Growth of frozen enrichment broths:

To test the incubation time appropriate for regrowth of cultures from frozen enrichment samples. Two 1.5 mL frozen enrichment samples from the hide of animal 10 were removed from the –70°C freezer and allowed to thaw at 4°C for 12 h. After thawing 1 mL of sample was added to each of three flasks containing 99 mL of mTSB-n and the flasks were incubated at 35°C. At 16, 19, 22, 24, 29 and 36 h, 1 mL samples were removed and prepared in the same manner as the initial samples were prepared for screening by PCR, using the *ehxA* primer. PCR was done after all samples were collected to determine if there were any differences due to incubation time in the ability of PCR to detect DNA from virulence genes.

3.11 Medium for isolation of EHEC:

Vancomycin-CCWBA was used to isolate enterohaemolysin-producing *E. coli* from faecal, hide, caecal and ground beef enrichment broths from animals 5, 10 and 15. Sterile sheep blood (Oxoid Inc., Nepean, ON) was prepared by measuring 100 mL of sheep blood into sterile polypropylene screw cap centrifuge bottles and centrifuging (Sorvall® RC-5B) at 1000 x *g* for 30 min to pellet the red blood cells. The supernatant was removed and the pellet was resuspended in 100 mL of 0.9% w/v phosphate buffered saline (PBS), pH 7.4, [NaCl 8 g (BDH), KCl 0.2 g (Fischer Scientific), Na_2HPO_4 1.44 g (Fisher Scientific), KH_2PO_4 0.24 g (Fisher Scientific) per 100 mL of mQ H_2O], and centrifuged (Sorvall® RC-5B) at 1000 x *g* for 30 min. This step was repeated four times. The final pellet was resuspended in 100 mL of PBS and stored at 4°C until required (not more than one month).

Vancomycin-CCWBA was prepared by mixing 33 g of Tryptose Blood agar base (Oxoid), 5 g Tryptose (BD), 5 g soluble starch (BD), 3 g agar (BD) and 441 mg of $CaCl_2$ (Fisher Scientific) in 970 mL of mQ H_2O (Lehmacher et al. 1998). This solution was heated to boiling, while stirring (15 min) and then it was autoclaved at 121°C for 20 min. The medium was then held at 50°C in a water bath for 2 h to ensure that the medium had cooled sufficiently prior to the addition of washed sheep blood. Washed sheep blood (3% final volume of blood) was added along with 30 mg/L of vancomycin, 3 mg/L of cefsulodin and 20 ng/L of cefixime. Vancomycin-CCWBA was mixed and dispensed into 150 x 15 mm petri plates (Fischer Scientific) (approximately 55 mL/plate) and allowed to cool and stored inverted at 4°C.

3.12 Isolation of EHEC using VCCWBA:

Frozen enrichment broths from the hide and caecal samples of animals 5, 10 and 15, which had tested positive for DNA from the *Stx*-2 and *ehxA* genes in the preliminary screening were thawed and reenriched in mTSB-n as described above. Faecal and ground beef samples for these three animals were also removed from the freezer and enriched in mTSB-n as described above. After incubation, enrichment broths were diluted (1/10) in 0.1% peptone water to a final concentration of approximately 10^{-5} CFU/mL and 100 µL of each dilution was spread plated on VCCWBA. Plates were allowed to dry for 15 min, inverted and placed in a GasPak® System 6 L anaerobic jar (BD) with three GasPak® Plus Anaerobic System envelopes with palladium catalyst (BD) and incubated for 36 h at 35°C. Agar plates with positive haemolytic (*E. coli* O157:H7 ATCC 43895) and negative non-haemolytic (*E. coli* ATCC 25922, *Y. enterocoltica* ATCC 23715 and *S. enterica* serovar Typhimurium ATCC 13311) controls were included in each jar. Agar plates streaked with gram-positive strains (*S. bovis* ATCC 15351, *L. monocytogenes* ATCC 15313 and *L. innocua* ATCC 33090) were also included in each jar to ensure that their growth was inhibited. Colonies with small turbid zones of haemolysis on VCCWBA (Lehmacher et al. 1998) were transferred into PCR strip-tubes containing 100 µL of mQ H_2O and the DNA was lysed as described above. Colonies were also transferred to a Microwell™ 96 well plate (Nunc-Nalgene) that contained 100 µL of mTSB-n. These plates were incubated at 35°C overnight, and glycerol (12.5% v/v) was added to each well prior to storage at -70°C.

Samples were plated onto Cefixime-Tellurite Sorbitol MacConkey agar (CT-SMAC)(BD) to isolate any *E. coli* O157:H7 that may have been present in the enrichment broths. Dilutions were prepared as above and plated onto CT-SMAC plates, which were incubated aerobically for 18 to 22 h at 35°C. Isolates from VCCWBA that were analyzed using PCR and had the characteristic *E. coli* O157:H7 virulence profiles were also spread onto CT-SMAC to test for the lack of sorbitol fermentation which is characteristic of most *E. coli* O157:H7 strains.

3.13 Reverse dot-blot hybridization (RDBH):

To compare PCR characterization and RDBH procedures the following laboratory strains, and strains isolated from the cattle production continuum were examined using both methods: *E. coli* strains O8:?, O103:H2, O153:H25, O163:NM, O26:H11, O5:NM O111:NM, O117:NM, O120:H5, O157:H7 (40), O157:H7 (54), O157:H7 (179), O157:H7 (181), O157:H7 (187), O157:H7 (188) from the Agriculture, Agri-Food Canada, Summerland Research Centre laboratory culture collection and *E. coli* strain numbers 5-1, 5-24, 5-16, 5-4, 5-10, 10-6, 10-10 isolated from hide enrichment broths and strain 10-4 isolated from ground beef enrichment broth at the AAFC Lacombe Research Centre. Briefly Oligonucleotide software version 6 was used to design *E. coli*-specific oligonucleotides. Oligonucleotides were synthesized with a reactive 5' amine group and they were applied directly to a preactivated nylon membrane (Immunodyne ABC. Pall, GlenCove, NY, USA). DNA extracted from pure cultures (FastDNA kit, Bio 101) was simultaneously amplified (GeneAmp PCR System 2400, Perkin-Elmer Applied Biosystems) and labeled with alkaline labile digoxygenin (DIG) using six primers [*eaeA*, *Stx*-1, *Stx*-2, *uidA*, *hlyA*, *uidA* other (the functional β-glucuronidase gene of *E. coli*)].

Dig-amplified samples were hybridized overnight at 52°C with the membrane-bound oligonucleotides and hybridized DNA was detected using anti-DIG antibody-alkaline phosphatase conjugate and CDP-star substrate (Roche Diagnostics).

3.14 Colony hybridization:

To test for an alternative method the isolation of colonies on VCCWBA followed by PCR screening of EHEC strains from the cattle production continuum, colony hybridization and slot-blot hybridization procedures were examined.

3.14.1 Preparation of membranes:

E. coli (ATCC 25922), *E. coli* O157:H7 (ATCC 43895), *Y. enterocolitica* (ATCC 23715) and *S. enterica* serovar Typhimurium (ATCC 13311) cultures were grown as above and diluted to approximately 10^{-7} CFU/mL (1/10 dilution in 0.1% w/v sterile peptone water). These cultures were mixed together to form a cocktail and plated onto Tryptic Soy Agar (TSA) plates (BD), which were incubated at 37°C for 18 to 20 h. After incubation the plates were held at 4°C for 2 h. Pure cultures of *E. coli* biotype 1 (ATCC 25922) and *E. coli* O157:H7 (ATCC 43895) were also prepared as above.

Hybond® nylon membranes (Amersham Pharmacia Biotech) were pre-cut so they were slightly smaller than petri plates. Membranes were labeled with a marker for orientation and interlaced in Whatman 3MM paper (VWR), wrapped in aluminum foil and sterilized by autoclaving at 121°C for 10 min. Membranes were cooled to room temperature overnight. Membranes were placed, labeled side down, onto TSA plates that had been cooled for 2 h. Membranes were marked with a needle to ensure that they could be realigned after hybridizations, and then lifted from plates. Membranes were placed colony side up on Whatman 3MM paper soaked with 10% w/v sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and allowed to stand for 5 min. This was followed by a sequence of treatments (5 min each) with a denaturing dolution [0.5 M NaOH (BDH), 1.5 M NaCl], neutralizing solution [1.5 mM NaCl, 0.5 M Tris-HCl pH 7.4 (Sigma-Aldrich)], and 2x SSC [3.0 M NaCl, 0.3 M Sodium Acetate (Sigma-Aldrich)] in succession. After treatments, membranes were placed on fresh Whatman 3MM paper and allowed to air dry for 30 min. Membranes were then interlaced in Whatman 3MM paper and placed in a Fisher vacuum oven (Fischer Scientific) at 80°C for 2 h. Membranes were removed from the oven and allowed to cool to room temperature. After cooling, the membranes were wrapped in aluminum foil and stored at 4°C until hybridization was done.

3.14.2 Preparation of probes:

DNA probes required for hybridization were made by amplifying specific sequences (*ehxA* and *uspA*) using PCR (Table 7). PCR amplification products were separated in 1.5% Ultrapure low melting temperature agarose (LMT; Bethesda Research Laboratories, Gaithersburg, MD) by submarine gel electrophoresis and viewed under UV light. Bands were excised with a razor blade and placed in a pre-weighed 1.5 mL Eppendorf microcentrifuge tube. Three millilitres of mQ H_2O per gram of gel was added to the tube. This solution was heated to 65°C for 5 min to melt the LMT. The sample was then mixed by inversion and stored at –20°C until labeling reactions.

Probes were labeled using a Stratagene Prime-it® Random Primer Labeling Kit (Stratagene Cloning System). The probe was removed from the freezer and allowed to thaw at room temperature. Twenty-five nanograms of DNA (9.6 μL) was added to 14.4 μL of mQ H_2O and 10 μL of random 9mer oligonucleotide primers. The reaction tube was heated in a boiling water bath for 5 min and centrifuged (Eppendorf 5415C) briefly at 16,000 x *g* to collect all liquid that had condensed on the cap. The reaction tube was held at 37°C, while 0.5x dCTP primer buffer (containing dATP, dGTP and dTTP), 50 μCi of α-^{32}P dATP (Amersham Pharmacia Biotech) and 5 U of Exo (-) Klenow enzyme were added. The reaction mixture was incubated at 37°C for 10 min and 2 µL of stop mix (0.5 M EDTA, pH 8.0) was added. The probe was stored at 4°C until required, for a maximum of 2 h.

3.14.3 Hybridization:

Membranes were removed from the 4°C incubator and allowed to equilibrate to room temperature. Membranes were soaked for 2 min in 6x SSC. Prehybridization solution (15 mL) [6x SSC, 5x Denhardts {0.5% Ficoll type 400 (BDH), 0.5% Polyvinylpyrrolidone (BDH) and 0.5% Bovine Serum Albumin Fraction 5 (Sigma)}, 0.5% (w/v) SDS, 100 µg/mL salmon sperm DNA (Life Technologies) and 50% (w/v) formamide (Sigma)] was added to a 150 x 35 mm hybridization tube (Thermo Hybaid, Ashford, Middlesex, UK) and heated in a hybridization incubator (series 942, Lab-Line Instruments Inc., Melrose Park, IL) to 42°C. A membrane was added to the tube containing preheated prehybridizaion solution and heated at 42°C for 2 to 3 h. Hybridization solution (prehybridization without Denhardts solution) was heated to 42°C while the membrane was prehybridizing. The radiolabeled probe was removed from the 4°C cooler and heated in a boiling water bath for 5 min and immediately submerged in an ice bath. After 3 h, the prehybridization solution was poured off the membrane and the warmed hybridization solution and probe were added to the tube with the prehybridized membrane and hybridized at 42°C for 16 to 20 h.

3.14.4 Washing and development of membranes:

After hybridization the membrane was washed using 50 mL of wash solution 1 [2x SSC, 0.1% SDS (w/v)] and shaken for 5 min at room temperature in a hybridization oven. This procedure was repeated once. The membrane was washed with wash solution

2 [1 x SSC, 0.1% SDS (w/v)] and shaken for 15 min at room temperature. Wash solution 3 (that had been preheated) [0.1% SSC, 0.1% SDS (w/v)] was added and the membrane solutions were heated at 65°C for 10 min. After washing, the presence of radioactivity on the membrane was checked using a hand-held Geiger counter. The membrane was sealed between sheets of plastic and loaded into an X-ray film cassette. In a dark room, Kodak Bio Max Film (Eastman-Kodak) was placed over the membrane and the cassette was wrapped in dark plastic. The cassette was stored at –70°C for at least 24 h. Films were developed using a Kodak M35A X-Omat Processor (Eastman-Kodak) according to directions.

A slight modification of the previoius procedure was done using a Microwell^{Tm} 96 well plate for culture growth. After growth in the microwell^{Tm} plate, a Nunc replication system 96-pin replicater (Nunc-Nalgene) was used to transfer cultures to an omni well tray (Nunc-Nalgene) containing TSA. After cultures were incubated at 37°C, they were transferred to membranes for hybridization as described previously.

3.15 Slot-blot hybridization:

3.15.1 Preparation of DNA:

Purified DNA isolated from *E. coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 43895), *Y. enterocolitica* (ATCC 23715), *S. enterica* serovar Typhimurium (ATCC 13311) and 20 strains (H1 to H20) isolated from the hide enrichment broths of animal 10. Strains isolated from animal 10 had been previously characterized using PCR. DNA was purified using the Wizard DNA purification kit. The DNA was denatured using 0.4 M NaOH, 10 mM EDTA and heated in a boiling water bath for 10 min. After cooling to room temperature, the DNA was neutralized by adding ice-cold ammonium acetate (BDH) pH 7.0 to a final concentration of 2 M.

3.15.2 Preparation of membranes:

Zeta-Probe® Blotting membranes (Bio-Rad) were moistened with mQ H_2O and set into the Bio-Dot (Slot-Blot) apparatus (Bio-Rad), according to the manufacturer's instructions, and attached to a vacuum apparatus. Any wells that were not used were sealed with taped to ensure the vacuum was even throughout. Wells were loaded with 500 µL of mQ H_2O and run through the membrane. Template DNA (400 µL) was loaded into the wells and run onto the membrane, and 500 µL of 0.4 M NaOH was added to fix the DNA to the membrane. The membrane was removed from the apparatus for hybridization.

3.15.3 Hybridization and washing:

Membranes were placed in hybridization tubes containing prewarmed hybridization solution [1 mM EDTA, 7% SDS (w/v), 0.5 M Na_2HPO_4, pH 7.2] for 5 min in hybridization incubator at 42°C. Fresh prewarmed hybridization solution was added to the tube and membranes were hybridized for 16 h. After hybridization, the hybridization solution was removed and wash solution A [1 mM EDTA, 40 mM Na_2HPO_4, pH 7.2, 5% SDS (w/v)] was added. The tube was shaken for 30 min at room temperature. Wash solution A was removed and wash solution B [1 mM EDTA, 40 mM Na_2HPO_4, pH 7.2, 1% SDS (w/v)] was added and the membrane was shaken for 30 min at 60°C. After washing step B, membranes were sealed in plastic and developed as described previously.

3.16 Randomly amplified polymorphic DNA (RAPD) analysis of E. coli isolates:

Random amplification of polymorphic DNA was used to test the relatedness of possible *E. coli* O157:H7 strains (H1 to H11 from hide enrichment broth from animal 10). Template DNA was prepared from H1 to H11 *E. coli* strains by isolating single colonies on VCCWBA, which were picked using sterile toothpicks and suspended in 100 μL of sterile mQ H_2O. Samples were boiled for 15 min followed by centrifugation in an Eppendorf Centrifuge 5417C (Brinkman) at 1,700 x *g* for 6 min. Random amplification of polymorphic DNA analysis of *E. coli* strains was performed according to the procedures described by Pacheco et al., (1996) with a few modifications. Briefly, 25 μL PCR mixture consisting of 5 μL of 1/100 diluted crude DNA, 2.5 mM $MgCl_2$, 0.8 nM 1254 decamer primer (5' –CCGCAGCCAA-3')(Sigma-Aldrich), 300 μM of each dNTP and 1 U *Taq* DNA polymerase was prepared. PCR amplification was performed in a Robocycler Infinity Thermocycler according to the following protocol, 1 cycle: 5 min each at 94°C, 36°C and 72°C, 10 cycles: 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, 20 cycles: 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. Amplified DNA fragments were separated on 1.8% agarose gels (90 volts for 2 hours) and stained with ethidium bromide for 45 min. A 100-bp DNA ladder was included as a molecular size marker. Stained gels were scanned digitally with the Kodak EDAS290 system and images were stored as tagged image format (TIFF) files. DNA patterns obtained with RAPD techniques were analyzed with Molecular Analyst Software, Fingerprinting version 1.61 (Bio-Rad Laboratories, Hercules, CA). Similarities between the DNA patterns of *E. coli* isolates based on band positions were determined using Dice similarity coefficient. A dendogram was constructed using unweighted pair group method using arithmetic averages (UPGMA) to reflect similarities in the matrix. *E. coli* isolates were grouped in types that were considered genetically related ($\geq$ 80% similarity) based on DNA patterns.

3.17 PCR-restriction fragment length polymorphism (RFLP) for analysis of the fliC gene:

The analysis of *fliC* gene encoding H antigen of *E. coli* was performed according to the procedure described by Fields et al. (1997), on the same strains analyzed by RAPD analysis (H1 to H11). Approximately 1.3 to 2.6 kb fragments of the *fliC* gene were amplified by PCR. The amplified fragments were digested with restriction enzyme *RsaI* at 37°C for 2 hours. The DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. A 100 bp DNA ladder was included as a molecular size marker.

3.18 Pulsed field gel electrophoresis:

Pulsed field gel electrophoresis (PFGE) was done on strains of *E. coli* isolated on VCCWBA from hide and caecal enrichment broths. Strains used were H10-14 to H10-17 from hide enrichment broths from animal 10, GB10-3, 10-11, 10-16 from ground beef enrichment broths from animal 10, H5-15, H5-25, H5-37, and H5-41 from hide enrichment broths from animal 5, and GB5-10 from ground beef enrichment broths from animal 5. Isolated *E. coli* strains were grown overnight on blood agar plates, then isolated colonies were picked from plates with sterile toothpicks and embedded in 0.8% low melting point agarose plugs (Invitrogen, Burlington, ON). Embedded *E. coli* plugs were lysed by overnight digestion with proteinase K (0.5 mg/mL) (Invitrogen) in 0.25 M EDTA (pH 9.5), 1% sodium lauroyl sarcosine (Sigma-Aldrich) at 50°C. A 1-mm slice of agarose was cut from the initial plug and digested with *Xba*-I (Invitrogen) for 2 h at 37°C. Each slice was rinsed in 0.5x TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) and embedded in a 1% agarose gel in 0.5x TBE for fragment separation. Digested DNA was subjected to PFGE for 24 h (initial switch time: 2.2 s, final switch time: 54.2 s, 6 volts/cm, included angle 120°). Bands were visualized by ethidium bromide staining and band patterns were photographed using a Kodak DC290 Zoom Digital Camera with Kodak 1D Image Analysis Software.

3.19 Identification of virulence factors through the beef cattle production continuum:

Figure 4 summarizes all procedures used for the identification of virulence factors through the beef cattle production continuum. Faecal, hide, caecal and ground beef enrichment broths from 30 head of cattle were characterize for the presence of virulence genes by PCR. Faecal samples from the animals after one and five months on pasture, one and four months in feedlot and caecal, hide, and ground beef enrichment broths were analyzed using PCR. Enrichment broths for each of the sample types (faeal, hide, caecal and ground beef) for a total of 210 enrichment broths were set up as described above. After overnight growth in mTSB-n, DNA was isolated and purified from each of the 210 enrichment broths using the Wizard® Genomic DNA purification kit (as described previously). PCR was then used to characterize each enrichment broth for the presence of the *E. coli uspA* gene. All enrichment broths that tested positive for the *uspA* gene were then analyzed by PCR for the presence of the four virulence genes (*stx*-1, *stx*-2, *ehxA*, and *eaeA*). Any enrichment broth that tested positive for the combination of *stx* virulence genes and *ehxA* and *eaeA* were also tested for the presence of the non-functional *uidA* gene of *E. coli* O157:H7.

Hide enrichment broths from the first ten animals slaughtered on March 21st, were prepared as above for the isolation of enterohaemolysin producing strains on VCCWBA plates. A maximun of 10 strains isolated on VCCWBA from each hide enrichment broth were then transferred using sterile toothpicks to 96 well microwell plate containing mTSB-n. Microwell plates were incubated for 20 h and *E. coli* strains were then

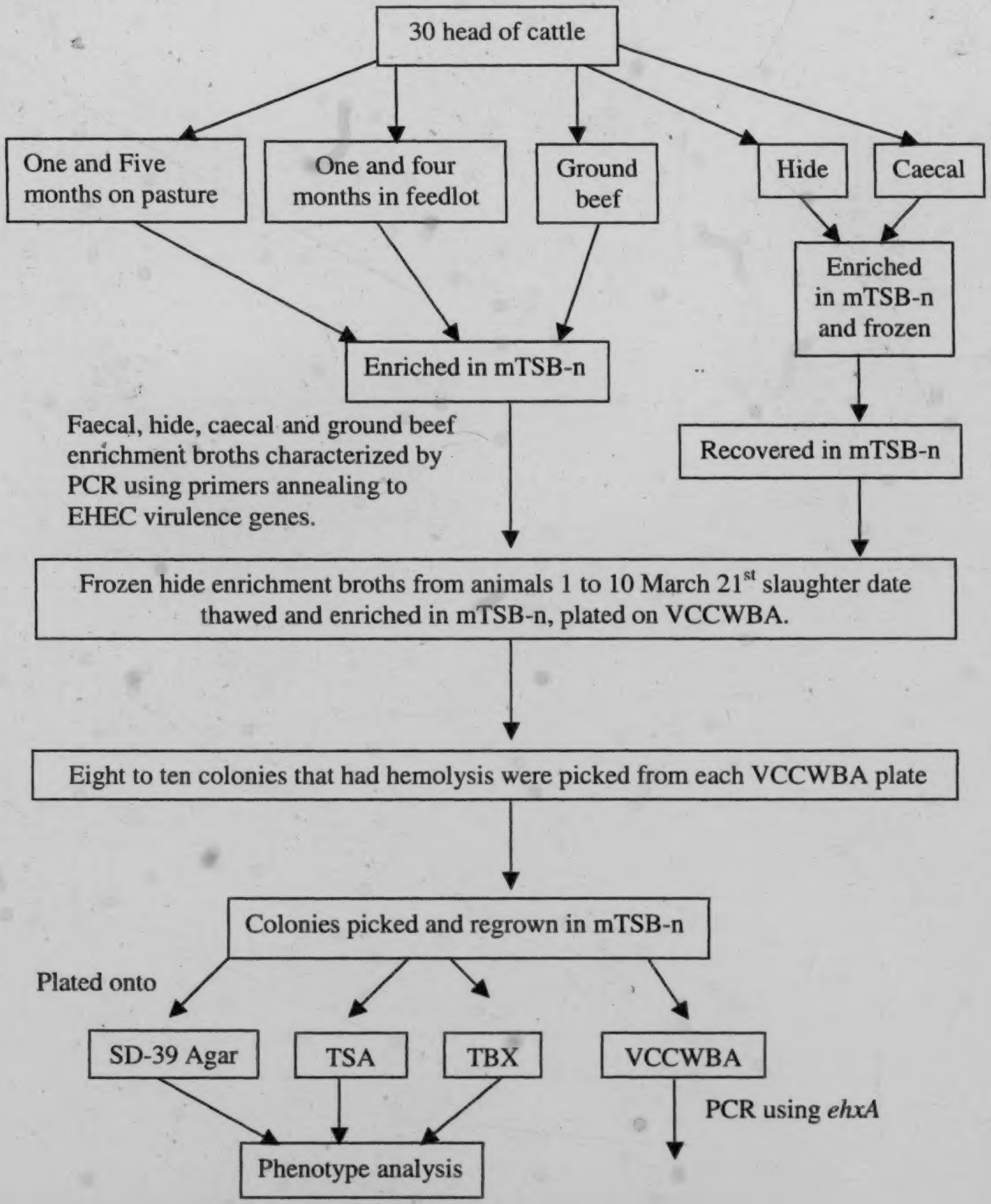

30 head of cattle
One and Five months on pasture
One and four months in feedlot
Ground beef
Hide
Caecal
Enriched in mTSB-n and frozen
Enriched in mTSB-n
Faecal, hide, caecal and ground beef enrichment broths characterized by PCR using primers annealing to EHEC virulence genes.
Recovered in mTSB-n
Frozen hide enrichment broths from animals 1 to 10 March 21st slaughter date thawed and enriched in mTSB-n, plated on VCCWBA.
Eight to ten colonies that had hemolysis were picked from each VCCWBA plate
Colonies picked and regrown in mTSB-n
Plated onto
SD-39 Agar
TSA
TBX
VCCWBA
PCR using *ehxA*
Phenotype analysis

Figure 4: Schematic diagram illustrating the procedures used in the characterization of EHEC virulence factors in faecal, hide, caecal and ground beef mTSB-n enrichment broths, through the beef cattle production continuum. mTSB-n, modified tryptic soy broth supplemented with novobiocin. VCCWBA, Vancomycin cefixime, cefsulodin washed sheep blood agar. TSA, tryptic soy agar. TBX, Tryptone bile agar with X-glucuronide.

transferred, using a 96-pin replicator onto omni-well agar plates containing selective medium SD-39 (QA Life Sciences, Inc. San Diego, CA), Tryptone Bile agar with X-glucuronide (TBX) (Oxoid), TSA and CT-SMAC. SD-39 and TBX plates were incubated at 44°C for 18 to 20 h; TSA plates were incubated at 37°C for 18 to 20 h. Strains were also transferred using the 96-pin replicator into 96 well microwell plates containing 1 mL of mTSB-n. Strains were incubated at 37°C for 20 h. DNA was then isolated from each strain using the Wizard® Genomic DNA purification kit as described previously. Each strain was then examined for the presence of the *ehxA* gene by PCR.

4 Results

4.1 Optimization of PCR:

Previously published PCR procedures for the detection of four EHEC virulence genes (*ehxA*, *stx*-1, *stx*-2 and *eaeA*), along with the non-functional *uidA* gene of *E. coli* O157:H7 and the *uspA* gene of *E. coli* were optimized for the Stratagene Robocycler Infinity thermal cycler. Initial trials used published parameters for all factors and then optimization of $MgCl_2$ and dNTP concentration, annealing temperature and annealing time. Final concentrations and conditions for each primer are listed in Table 7. Examples of the products produced during optimization reactions are shown in Figure 5 (Temperature) and Figure 6 ($MgCl_2$ and dNTP).

Figure 5 shows the results for the effect of temperature on gene amplification. For the *eaeA* primer a temperature of 65°C was chosen since a bright band along with faint non-specific bands were obtained. Temperatures of 59°C or 69°C produced less prominent bands and a greater amount of non-specific banding occurred. After temperature gradients were done, gradients of $MgCl_2$ and dNTPs were tested at 65°C to determine the most appropriate combination. The best combinations were 3.5 mM $MgCl_2$ and 0.25 mM dNTPs for the *eaeA* primer (Figure 6; lane 8). These combinations gave the brightest banding with the least amount of non-specific binding. The optimization was completed for all six primer pairs. The best results were produced using $MgCl_2$ concentrations of 3.0 mM and 0.25 mM dNTPs for the *stx*-1, *stx*-2, *ehxA*, *uidA* and *uspA*, where as 3.5 mM $MgCl_2$ and 0.25mM dNTPs were used for amplification of the *eaeA* primer. Figure 7 shows the amplification products of all six primer pairs.

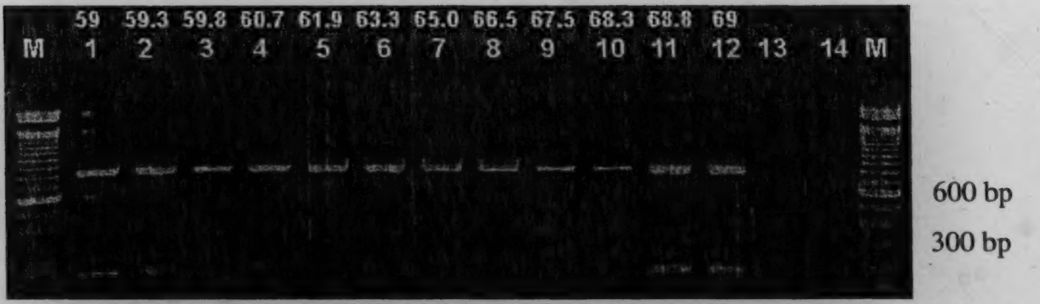

59 59.3 59.8 60.7 61.9 63.3 65.0 66.5 67.5 68.3 68.8 69
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
600 bp
300 bp

Figure 5: Effect of PCR reaction tempertature on amplification of *eaeA* PCR products from *E. coli* O157:H7 (ATCC 43895) purfied genomic DNA. Numbers above lanes 1 to 12 are annealing temperatures used for products run in each lane. Lanes marked with an M were 100 bp DNA ladder; PCR products separated in lane 1 and 12 were from PCR reactions run at 59°C and 69°C, respectively. Lanes 13 and 14 are negative controls, were template DNA was replaced with mQ H_2O annealment at 65°C.

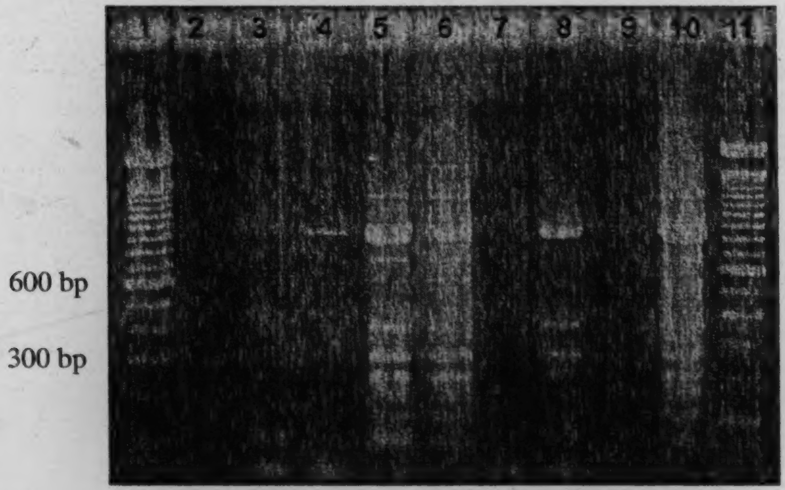


Figure 6: Effect of dNTP and $MgCl_2$ concentration on amplification of *eaeA* PCR products from *E. coli* O157:H7 (ATCC 43895) purfied genomic DNA. All lanes were amplified with an annealing temperature of 65°C. Lanes 1 and 11 were a 100 bp DNA ladder. Lanes 2 through 6 were run with 0.5 mM dNTP and 2.75, 3.0, 3.25, 3.5 or 3.75 mM $MgCl_2$, respectively. Lanes 7 through 10 were run with 0.25 mM dNTP concentration and 3.25, 3.5, 3.75 or 4.0 mM $MgCl_2$, respectively.

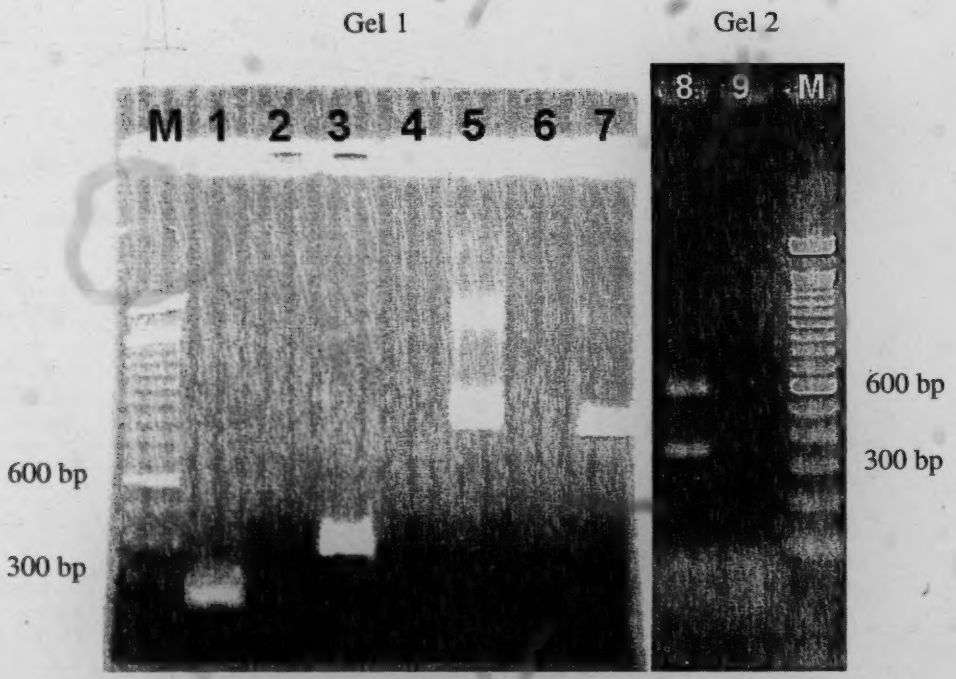


Figure 7: PCR amplification of the *uidA, ehxA, uspA, eaeA, stx*-1, and *stx*-2 genes to be used in the study of EHEC virulence genes in *E. coli* strains isolated from cattle at different steps of the production process. Gel #1 (Lanes 1 to 7) shows the *uidA, ehxA, uspA* and *eaeA* amplification products (lanes 1, 3, 5, and 7, respectively) from *E. coli* O157:H7; Gel #2 (lanes 8 and 9) shows the amplification products for the *stx*-1 and *stx*-2 primer pairs from *E. coli* O157:H7. Lanes 2, 4, 6 and 9 are all negative controls and lanes marked M were a 100 bp DNA ladder.

4.2 Sequence analysis:

Sequencing of gene fragments amplified during PCR analysis was done to ensure that the products of PCR amplification were correct. Sequences were analyzed for percent relatedness to gene sequences published in GeneBank. Each sequence had high homology with published sequences. The *ehxA* (accession number ECO12572) gene had 100% homology at the nucleotide level, whereas the *uspA* (AF346731), *uidA* (AF305917), *stx*-2 (AY143336), and *eaeA* (EW32312) genes had >99% homology, and the *stx*-1 (AF461169) gene had >98% homology. These results indicate that the PCR was amplifying the target sequences correctly.

4.3 Initial screening of enrichment broth for the presence of *ehxA* and *stx*-2:

After optimization of PCR had been completed, DNA isolated from enrichment broths obtained from hide and caecal samples from the March 21st and April 3rd slaughter days were analyzed for the presence of the *ehxA* and *stx*-2 genes, (Table 8 and Figure 8). All enrichment broths from the hide samples obtained from animals slaughtered on March 21st (animals 1 to 15) were positive for DNA from the *ehxA* gene, whereas 11 of the 15 caecal samples were positive. DNA from the *stx*-2 gene was detected in 14 of the 15 hide enrichment broths and in nine of the 15 caecal enrichment broths. For the second slaughter on April 3rd (animals 16 to 30) only three out of 15 of the enrichment broths from the hide samples and one out of 15 enrichment broths from caecal samples were positive for the *ehxA* gene, whereas one of the 15 enrichment broths and two of the 15 enrichment broths from hide and caecal samples respectively, were positive for the *stx*-2 gene. Figure 8 shows the percentage of enrichment broths that tested positive for the presence of the two virulence genes. The overall trend showed that the hide and caecal

Table 8: Detection of DNA by PCR of the *ehxA* and *stx*-2 genes in mTSB-n[a] enrichment broths for hide and caecal samples obtained from animals slaughtered at the Lacombe Research Centre on March 21st (animals 1 to 15) and April 3rd (animals 16 to 30).

Animal[b]	*ehxA* gene		*stx*-2 gene	
	Hide[c]	Caecum[d]	Hide	Caecum
1	+	-	+	-
2	+	+	+	+
3	+	-	+	+
4	+	+	+	+
5[e]	+	+	+	+
6	+	-	+	-
7	+	+	+	+
8	+	+	+	+
9	+	+	+	-
10[e]	+	+	+	+
11	+	+	+	-
12	+	+	+	+
13	+	-	+	-
14	+	-	-	-
15[e]	+	+	+	+
17	+	-	-	-
18	+	-	-	+
19	+	-	-	-
23	-	-	+	+
30	-	+	-	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] Data for samples that were negative for both genes are not shown. This includes samples from animals 16, 20 to 22, and 24 to 29. Original animal numbers are listed in Appendix A.

[c] Hide sponge samples taken at time of slaughter.

[d] Caecal samples collected just after slaughter.

[e] Enrichment broths for samples from animals 5, 10 and 15 were chosen for future characterization.

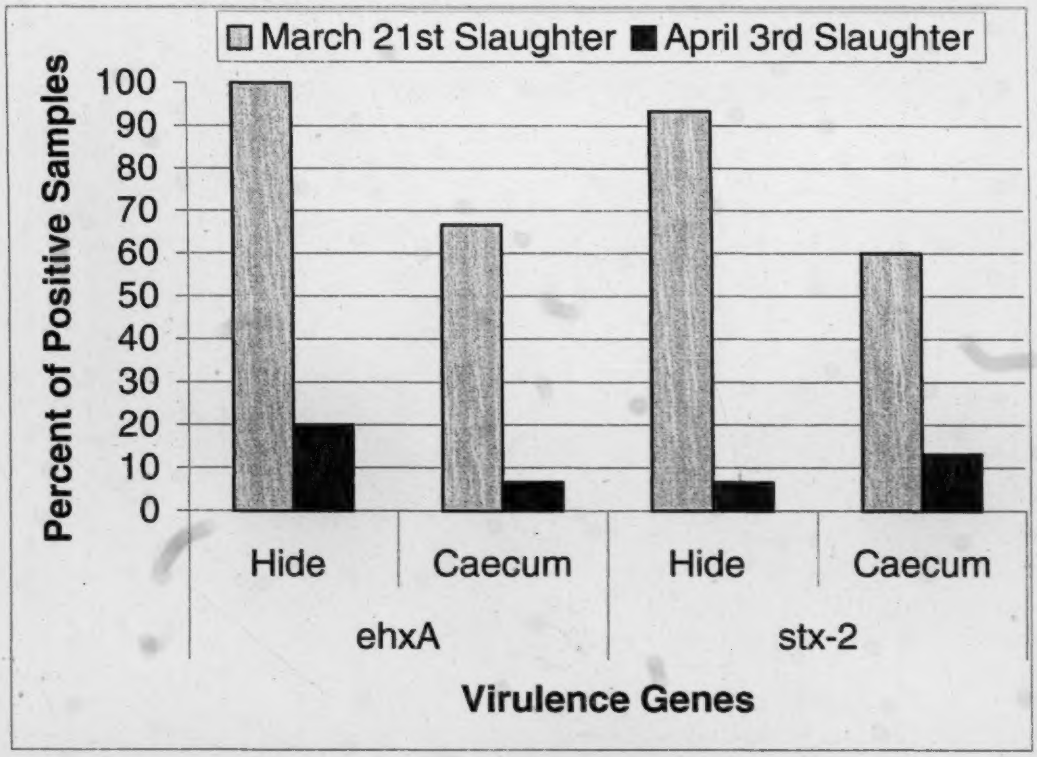


Figure 8: Percentage of enrichment broths prepared from hide and caecal samples obtained from beef cattle slaughtered on two different processing days that tested positive by PCR analysis for the presence of virulence genes *ehxA* and *stx*-2.

samples obtained from animals slaughtered on March 21st had a greater percentage of positive samples compared to those obtained from animals slaughtered on April 3rd. Also for the first slaughter, there seemed to be a big difference in positive enrichment broths from hide samples compared to enrichment broths from caecal samples. The percentage of hide and caecal enrichment broths that tested positive were similar for animals from the second slaughter. Based on results from the initial screening for the presence of two virulence genes, samples from animals 5, 10 and 15 were selected for further isolation and characterization of EHEC. These were chosen because both enrichment broths from the hide and caecal samples from these animals were positive for the two virulence genes.

4.4 Recovery of *E. coli* in frozen-enrichment broth from the hide of animal 10:

After the initial screening had been done, frozen samples from the hide of animal 10 that had been enriched in mTSB-n were regrown in fresh mTSB-n to determine the incubation times required for the samples to test positive using PCR to detect the presence of DNA from the *ehxA* gene. Samples that were incubated for 16 and 19 h tested positive. After incubation for 22 and 24 h, two of the three cultures were positive, while none of the cultures incubated longer than 29 h tested positive. Based on these results, frozen enrichment broths from samples obtained from animal 5, 10 and 15 were incubated in mTSB-n for 16 to 20 h for use in PCR assays.

4.5 Isolation and characterization of EHEC from animals 5, 10 and 15:

For each of the three animals, hide and caecal enrichment broths were thawed, while faecal and ground beef samples were thawed. After each sample was thawed it was enriched in mTSB-n and individual colonies were isolated, using the characteristic enterohaemolysin phenotype on VCCWBA. All colonies that had the enterohemolytic phenotype were characterized by PCR for the presence of *stx*-1, *stx*-2, *ehxA*, *eaeA*, *uspA* and *uidA* genes. The hide sample from animal 10 had 20 isolates that exhibited the enterohemolytic phenotype on VCCWBA. Table 9 indicates the genes that were present in each isolate obtained from the hide of animal 10. This is an example of how the isolates obtained at all stages of processing were analyzed (faecal, hide and ground beef sample) for each of the three animals (see Appendix B for complete listings of the characteristic from all strains from all samples).

Table 10 summarizes the PCR analysis of the colonies isolated from all three animals at each of the three sample times examined. For animal 5 there were 32, 41, and 31 strains isolated from the faecal, hide and ground beef samples, respectively, that were positive for enterohaemolysin production on VCCWBA (Table 9 and 10). Of these, 23/32 faecal, 41/41 hide and 23/31 ground beef strains were also positive for the *uspA* gene. From the strains isolated from faecal samples, only one was positive for *uspA* and another virulence factor (*ehxA*). There were 11 strains from the hide that were positive for the *uspA* and *ehxA* genes, and 17 strains were positive for *uspA*, *ehxA*, and *eaeA* genes. There was only one strain from the ground beef that was positive for *uspA* and *ehxA*, and one strain contained both the *uspA* and *stx*-2 genes.

Enrichment broths prepared from faceal, hide, and ground beef samples obtained from animal 10 (Table 10) had 27/32, 20/20 and 12/18 strains that were positive for the *uspA* gene, respectively. Of the 27 strains from faecal samples that were positive for the *uspA* gene, seven were also positive for the *ehxA* gene and another was positive for the *eaeA* gene. For the 20 strains isolated from the hide samples, 11 were positive for all

Table 9: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of hide samples obtained from animal 10 that had been plated onto VCCWBA[b].

Animal 10	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene
Isolate #	*uspA*	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*
H10-1	+	+	+	+	+	+
H10-2	+	+	+	+	+	+
H10-3	+	+	+	+	+	+
H10-4	+	+	+	+	+	+
H10-5	+	+	+	+	+	+
H10-6	+	+	+	+	+	+
H10-7	+	+	+	+	+	+
H10-8	+	+	+	+	+	+
H10-9	+	+	+	+	+	+
H10-10	+	+	+	+	+	+
H10-11	+	+	+	+	+	+
H10-12	+	-	-	-	-	-
H10-13	+	+	-	-	-	-
H10-14	+	+	-	+	-	-
H10-15	+	+	+	-	-	-
H10-16	+	+	+	-	-	-
H10-17	+	+	+	-	-	-
H10-18	+	-	-	-	-	-
H10-19	+	+	-	-	-	-
H10-20	+	-	-	-	-	-

[a] mTSB-n, Modified tryptic soy broth supplemented with novobiocin.
[b] VCCWBA, Vancomycin, cefixime, cefsulodin, washed sheep blood agar.

Table 10: PCR characterization of *E. coli* stains isolated from the mTSB-n[a] enrichment broths plated onto VCCWBA[b] for the faecal, hide and ground beef samples from animals 5, 10 and 15.

Animal	Sample	Date[c]	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene
			uspA	*eaeA*	*stx*-1	*stx*-2	*ehxA*	*uidA*
5	Faecal	Feb 14/00	23[d]	0	0	1	1	0
	Hide	March 21/00	41	21	0	0	30	0
	Ground Beef	March 24/00	23	0	0	1	1	0
10	Faecal	Feb 14/00	27	1	0	0	7	0
	Hide	March 21/00	20	11	11	12	17	11
	Ground beef	March 24/00	12	1	0	4	4	0
15	Faecal	Feb 14/00	28	3	0	0	8	0
	Hide	March 21/00	21	3	2	2	11	0
	Ground beef	March 24/00	0	0	0	0	0	0

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.
[b] VCCWBA, vancomycin, cefixime, cefsulodin, washed sheep blood agar.
[c] Sample collection date.
[d] Total number of isolates positive for specific markers for each sample type.

virulence genes and the *uidA* gene. These were tentatively identified as *E. coli* O157:H7. There were five other strains that were positive for *uspA* and *ehxA* genes, and one that had the *stx*-2 and *uspA* genes.

The *uspA* gene was detected in 28/32 and 21/24 of the strains isolated from the enrichment broths prepared from faecal and hide samples respectively, obtained from animal 15 (Table 10). No strains with enterohaemolysis activity were isolated from the ground beef prepared from animal 15. Of the 28 strains that were positive for the *uspA* gene, two were also positive for *ehxA* and *eaeA* genes, and another four were positive for the *ehxA* gene. The hide from animal 15 had six strains that were positive for the *uspA* and *ehxA* genes, and one strain was positive for *eaeA*, *stx*-2 and *ehxA*, one strain was positive for *stx*-1 and *ehxA*, one strain was positive for *ehxA* and *eaeA* and one strain was positive for the *eaeA* gene.

In all cases, the highest percentage of strains of *E. coli* were isolated from the hide. Strains from the hide also carried the greatest number of isolates that had the greatest number of multiple virulence genes, whereas very few strains from faecal and ground beef samples were positive for any virulence factors. These methods were very time consuming and there were a large number of strains that demonstrated the production of enterohaemolysin on VCCWBA but were not positive for the *ehxA* gene when analyzed using PCR. This apparent lack of specificity of the VCCWBA medium resulted in the testing of colonies that were not truly enterohaemolytic *E. coli*.

4.6 Comparison of PCR and RDBH for detection of EHEC virulence genes:

PCR results were compared to results obtained by RDBH to test the ability of both methods to detect EHEC virulence genes. There was excellent agreement between the two methods for the detection of the *uidA* other (detecting *E. coli*) and *uspA*, and also the detection of the non-functional β-glucuronidase (*uidA* genes) and the *ehxA* gene in strains of *E. coli* O157:H7. However, of the 24 strains tested, four strains were positive for *ehxA* by PCR and were negative by RDBH. Two of the 24 strains that were negative for the presence of the *eaeA* gene by PCR analysis were positive by RDBH and two of the 24 strains that were positive by RDBH were negative by PCR analysis. PCR detected the presence of *stx*-1 or *stx*-2 genes in four or five of 24 strains, respectively, that were not detected by RDBH (Table 11, and Table 12).

Sequence analysis of the *stx*-1 and *stx*-2 PCR amplification products was done to ensure that the correct regions of these genes were amplified during PCR. The sequences obtained from PCR amplification products matched those published for *stx*-1 and *stx*-2 genes indicating that discrepancies in the *stx* analysis was due to incorrect RDBH results. Comparison of these methods revealed that PCR was a sensitive procedure that correctly identified the EHEC virulence genes present in strains of *E. coli*.

4.7 Colony and slot-blot hybridization:

Colony hybridizations were done using the *uspA* and *ehxA* PCR amplification products as probes. Testing was done using pure cultures of *Y. enterocolitica* (ATCC 23715), *S. enterica* serovar Typhimurium (ATCC 13311), *E. coli* biotype 1 (ATCC 25922), and *E. coli* O157:H7 (ATCC 43895). Colonies were grown and probed to determine if *E. coli* O157:H7 could be differentiated from the other strains using the specified probes. Agar plates with a mixture of colonies of *Y. enterocolitica*, *S.enterica* serovar Typhimurium, *E. coli* biotype 1 and *E. coli* O157:H7 were probed with each of the two probes. There was no distinct indication that colony hybridization could

Table 11: Results for PCR analysis of marker genes and EHEC virulence genes in selected strains of *E. coli* of different serotypes.

Strain	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*
O157:H7	+	+	+	+	+	+
O8:?	+	-	-	+	-	-
O103:H2	+	+	+	-	+	-
O153:H25	+	+	+	+	+	-
O163:NM	+	+	+	-	-	-
O5:NM	+	+	-	-	-	-
O26:H11	+	+	+	-	+	-
O111:NM	+	+	+	+	+	-
O157:H7	+	+	-	-	+	+
O120:H5	+	-	-	-	-	-
O117:NM	+	-	+	-	-	-
O157:H7	+	+	+	+	+	+
O157:H7	+	+	+	+	+	+
O157:H7	+	+	+	+	+	+
O157:H7	+	+	+	+	+	+
10-4 GB	+	-	-	+	-	-
5-1 Hide	+	+	-	-	+	-
5-24 Hide	+	+	-	-	+	-
5-16 Hide	+	+	-	-	+	-
5-4 Hide	+	+	-	-	-	-
5-10 Hide	+	+	-	-	-	-
10-6 Hide	+	+	+	+	+	+
10-7 Hide	+	+	+	+	+	+
10-10 Hide	+	+	+	+	+	+

Table 12 Results for reverse dot-blot hybridization analysis of marker genes and EHEC virulence genes in selected strains of *E. coli*.

Strain	*E. coli* Marker	EHEC Virulence Genes				B-glucuronidase gene
	uidA (other)	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*
O157:H7	+	+	+	+	+	nd[a]
O8:?	+	-	-	+	-	nd
O103:H2	+	+	+	-	+	nd
O153:H25	+	+	-	+	-	nd
O163:NM	+	-	+	-	-	nd
O5:NM	+	+	-	-	-	nd
O26:H11	+	+	+	-	+	nd
O111:NM	+	+	+	+	+	nd
O157:H7	+	+	-	-	+	nd
O120:H5	+	-	-	-	-	nd
O117:NM	+	-	-	-	-	nd
O157:H7	+	+	+	-	+	nd
O157:H7	+	+	+	-	+	nd
O157:H7	+	+	-	-	+	nd
O157:H7	+	+	-	-	+	nd
10-4 GB	+	-	-	-	-	-
5-1 Hide	+	+	-	-	+	+
5-24 Hide	+	+	-	-	+	-
5-16 Hide	-	-	-	-	-	-
5-4 Hide	+	+	-	-	+	-
5-10 Hide	-	-	-	-	-	-
10-6 Hide	+	+	+	+	+	+
10-7 Hide	+	+	+	+	+	+
10-10 Hide	+	+	+	+	+	+

[a] nd, Not determined

differentiate among striains of the different enteric bacteria used in this study (Figure 9). None of the hybridized colonies showed a distinct radioactive mark, using either the *uspA* or the *ehxA* probe. An attempt to use colony hybridization to differentiate between *E. coli* O157:H7 (ATCC 43895) and other enteric pathogens was done by inoculating specific colonies onto the agar plates used for colony hybridization. Again colony hybridization was not able to differentiate between *E. coli* (ATCC 25922) and *E. coli* O157:H7 (ATCC 43895) from the other two organisms (Figure 10). These results indicated that colony hybridization could not be used as a screening method for identification of EHEC in mixed bacterial populations.

Slot-blot hybridization was done to determine if purified DNA from strains of E. coli would give a distinct signal that would allow identification of EHEC. DNA from strains known to be uspA and ehxA positive were used including E. coli (GGG8 to 10), E. coli O157:H7 (ATCC 43895) and S. enterica serovar Typhimurium as well as strains isolated from the hide of animals 5 and 10 (H10-1 to H10-20, and H5-1 to H5-4). Although the probe bound to the genomic DNA of stains of E. coli H10; 1 to 4, 6 to 9, 12, 13, 14B, 15, 16, 18 to 20, H5-20, E. coli O157:H7 (ATCC 43895) and E. coli GGG8 to 10 (Figure 9), there was background activity for the strains which were negative for the uspA gene (spots 21, 22 and 23, Figure 11) and the *ehxA* gene (data not shown). There were also weak hybridization reactions for sample 5, 10, 11, 17 and 24, which were previously determined by PCR to contain the *uspA* gene. The slot-blot hybridization procedure is a possibility for characterizing potentially pathogenic *E. coli* strains, however, this method is very time consuming and labour intensive.

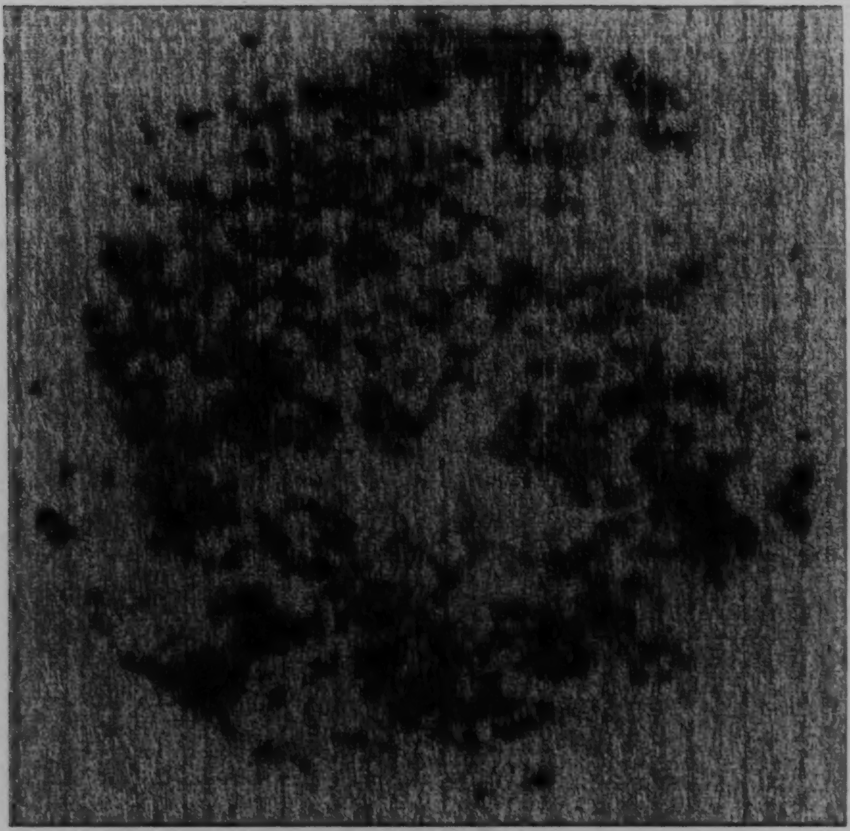


Figure 9: Colony hybridization of a spread plate containing *E. coli* (ATCC 25922) and *E. coli* O157:H7 (ATCC 43895) using a ^{32}P labeled *uspA* probe.

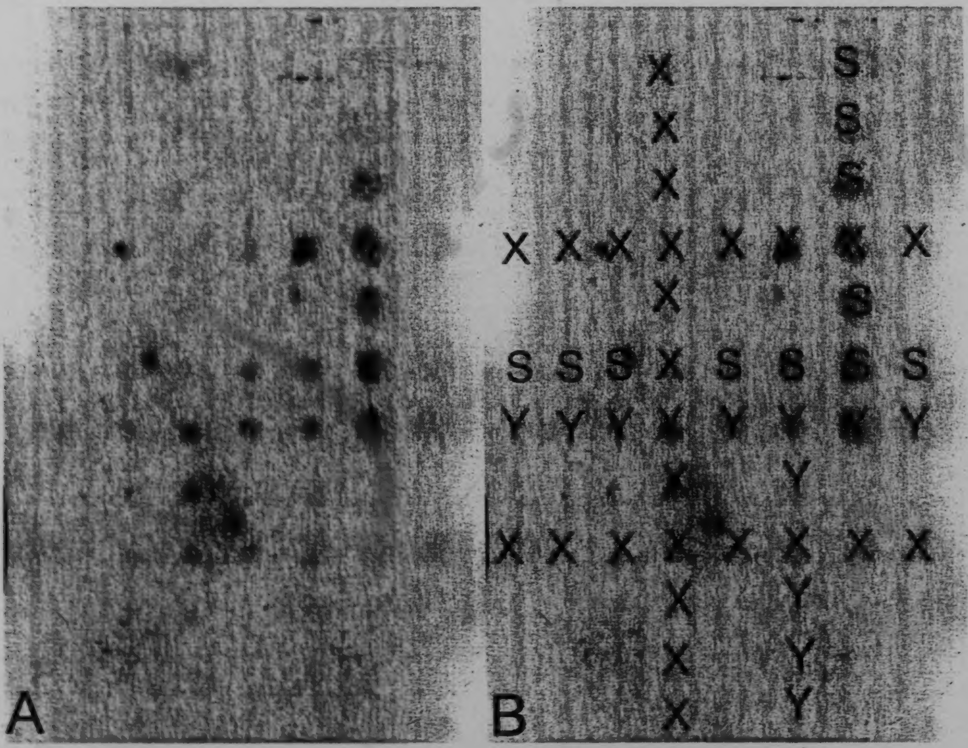

X S
X S
X S
X X X X X X S X
X S
S S S X S S S S
Y Y Y X Y Y X Y
X Y
X X X X X X X X
X Y
X Y
X Y
A
B

Figure 10: Colony-blot hybridization of *E. coli* O157:H7 (ATCC 43895), *S. enterica* serovar Typhimurium, (ATCC 13311) and *Y. enterocolitica* (ATCC 23715) using a ^{32}P labeled *ehxA* probe. Membrane A shows hybridizations membrane. Membrane B shows where the different colonies were on the membrane, S (*S. typhimurium*),Y (*Y. enterocolitica*) and X (*E. coli* O157:H7).

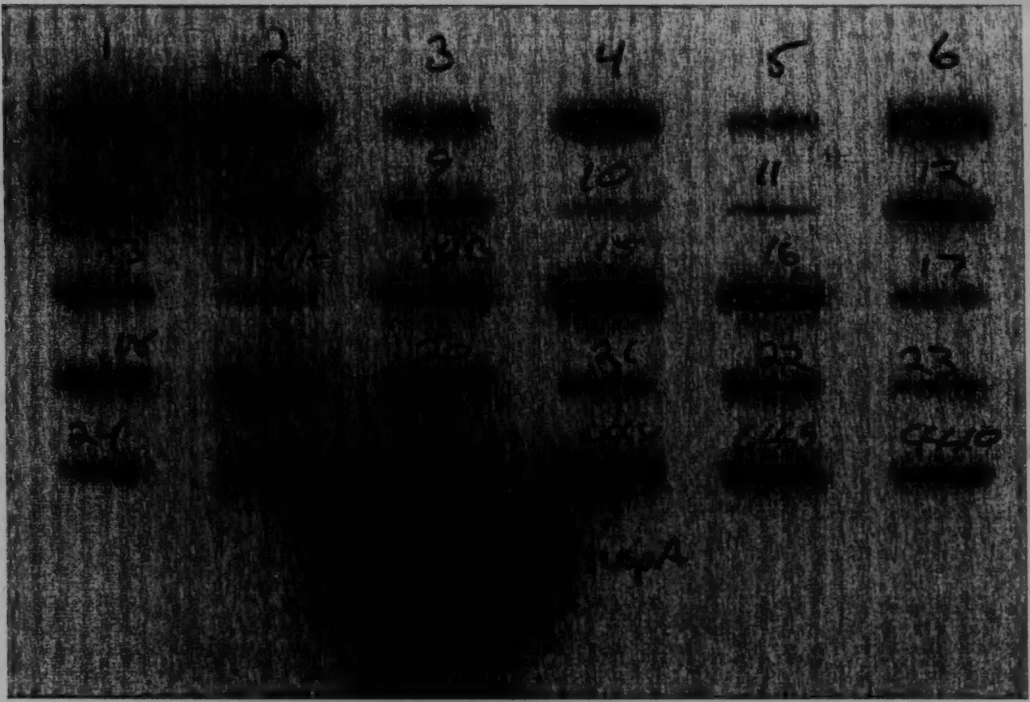

1
2
3
4
5
6
9
10
11
12
13
15
16
17
18
21
22
23
24
GGG9
GGG10
uspA

Figure 11: Slot-blot hybridization of purified genomic DNA from *E. coli* O157:H7 (ATCC 43895), *S. enterica* serovar Typhimurium, *E. coli* biotype 1 (GGG8 to GGG10), the PCR amplification product for the *uspA* DNA fragment (*uspA*), and strains isolated from the hides of animals 5 and 10) using a ^{32}P labeled *uspA* probe. 1 to 24, DNA isolated from H10-1 to H10-20 and H5-1 to H5-4 respectively). Sal, DNA isolated from *S. enterica* serovar Typhimurium. O157, DNA isolated from *E. coli* O157:H7 (ATCC 43895),

4.8 Analysis of *E. coli* strains obtained from hide and ground beef enriched broths by RAPD, PCR-RFLP and PFGE analysis to examine genetic relatedness:

Random amplification of polymorphic DNA analysis was used to examine the relatedness of possible *E. coli* O157:H7 strains, H10-1 to H10-11, obtained from enrichment broths from hide samples plated onto VCCWBA. Banding patterns created during RAPD analysis for all 11 (Figure 12) strains were the same, which indicates that all 11 strains were related. Polymerase chain reaction-restriction fragment length polymorphism analysis of the same 11 isolates revealed they all had the same flagellar antigen (*fliC*) genes (Figure 13), which were indicative of the O157:H7 flagellar antigen.

Pulsed field gel electrophoresis was done to determine if *E. coli* stains possessing virulence genes isolated at different stages in the beef cattle production could be tracked. Twelve strains were examined that had been isolated from enrichment broths from hide and ground beef samples from animal 5 and 10 (Figures 14 and 15). Four distinct electrophoresis types (ET) were observed. Electrophoresis Type 1 included 3 strains that were >90% related. All three strains were from the hide of animal ten (H10-15 to 17) possessed the *ehxA* gene. Electrophoresis Type 2 had a single strain isolated from the hide of animal 5 and it possessed the *ehxA* and *eaeA* genes. Type 3 was the largest ET, which included 6 strains that were all >90% related. Three strains were isolated from ground beef of animal 10 (GB10-3, 11 and 16), two strains were isolated from the hide of animal 5 (H5-1 and 25) and the final strain was isolated from the ground beef of animal 5 (GB5-10). The three strains isolated from the ground beef of animal 10 were positive for the *ehxA* gene. The two strains isolated from the hide of animal 5 were positive for the *ehxA* and *eaeA* genes. All the ground beef isolates from animal 5 were positive for the

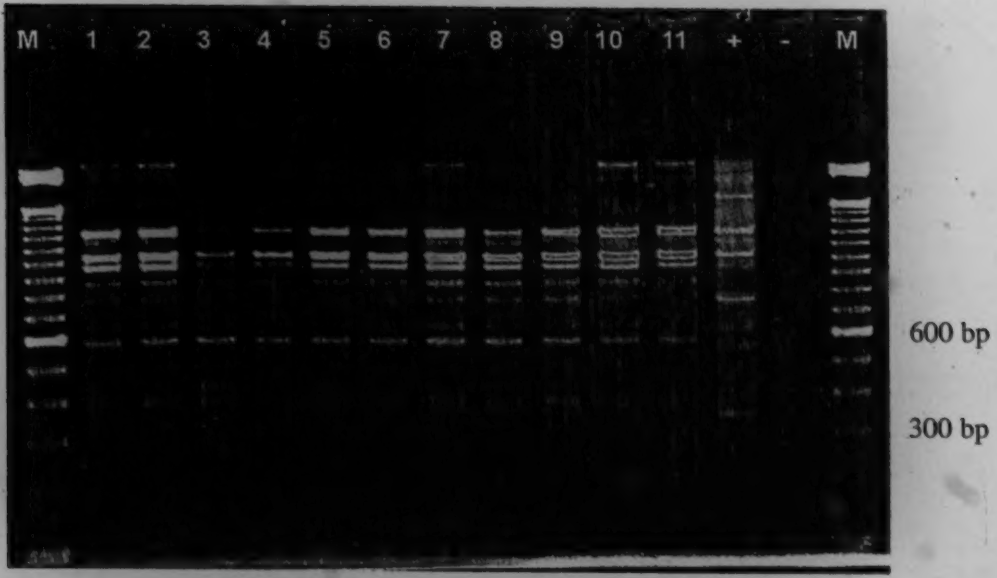


Figure 12: Randomly amplified polymorphic DNA analysis of DNA from *E. coli* strains isolated from hide samples from animal 10 enriched in mTSB-n. Lanes with an M were 100 bp ladders. Lane with a + or – were positive and negative controls respectively. Lanes 1 to 11 are amplification products from probable *E. coli* strains isolated from the hide of animal 10.

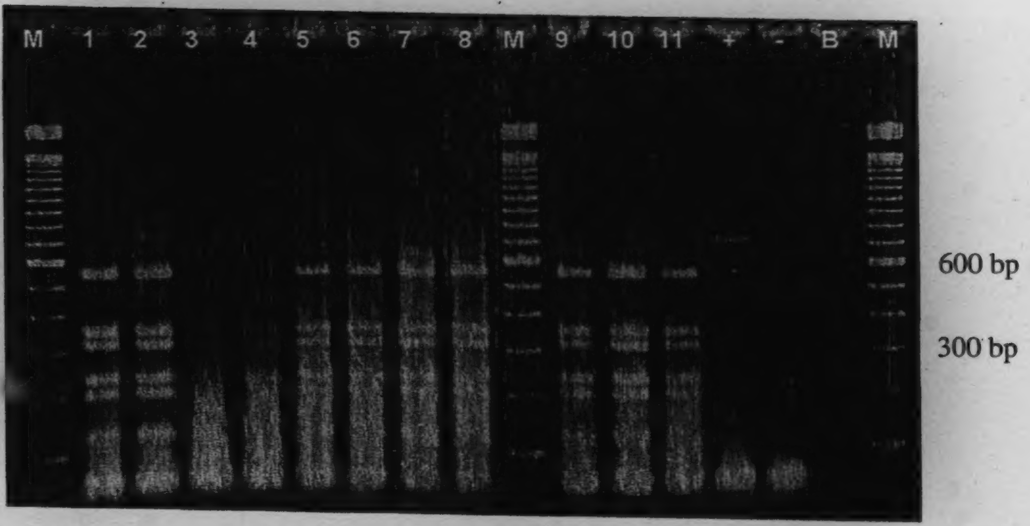


Figure 13: Polymerase chain reaction-restriction fragment length polymorphism analysis of DNA from *E. coli* strains isolated from hide samples from animal 10 enriched in mTSB-n. Lanes marked with a + or – were positive and negative controls, respectively. Lanes marked B were blank. Lanes marked M were 100 bp DNA ladder. Lanes 1 to 11 were *Rsa1* digestions profiles from PCR amplified *fliC* gene of the probable *E. coli* O157:H7 strains isolated from the hide of animal 10.

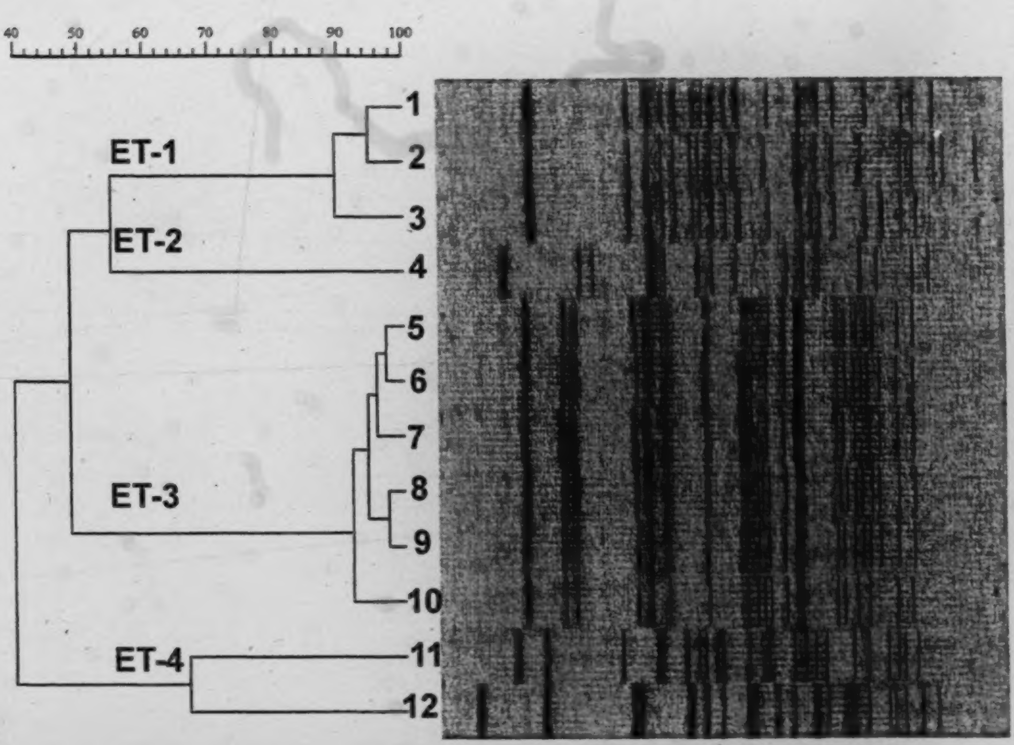


Figure 14: Pulsed field gel electrophoresis analysis of strains of *E. coli* isolated from the hide and ground beef from animals 5 and 10. Strains are grouped according to their percent relatedness (Dice Coefficient). Lanes 1 to 3 and 11 were all strains of *E. coli* isolated from the hide of animal 10, lanes 4, 8, 10, 12 were all strains of *E. coli* isolated from the hide of animal 5, lanes 5 to 7 were all strains of *E. coli* isolated from the ground beef of animal 10, and lane 9 had a strain of *E. coli* isolated from the ground beef of animal 5. ET, Electrophoretic type.

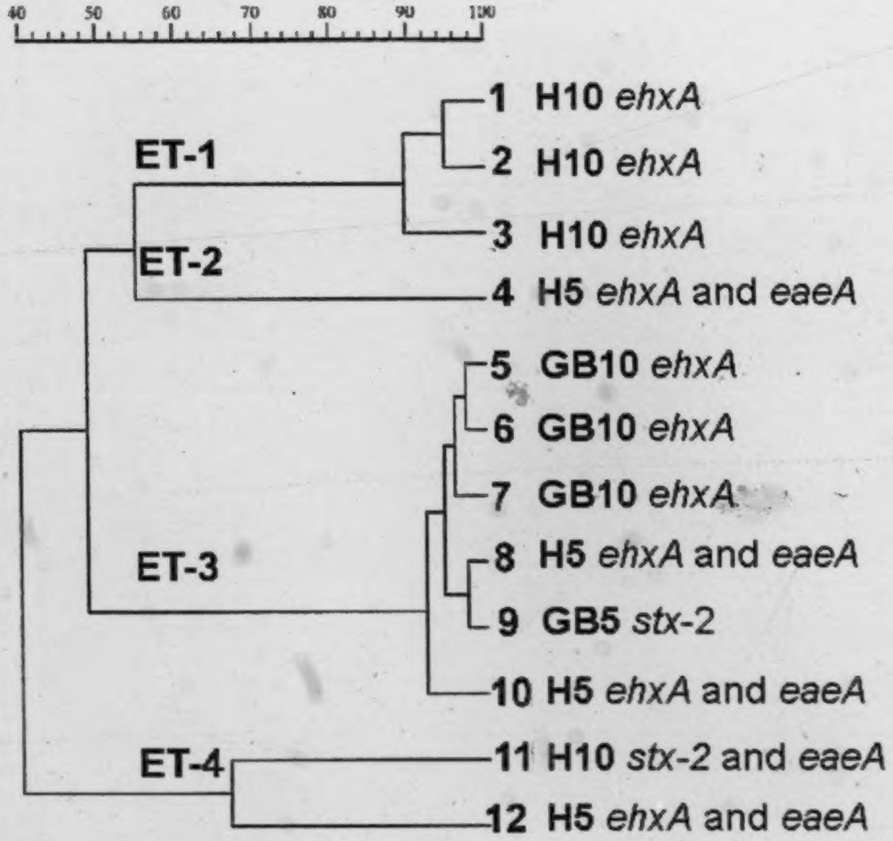

40
50
60
70
80
90
100
ET-1
ET-2
ET-3
ET-4
1 H10 *ehxA*
2 H10 *ehxA*
3 H10 *ehxA*
4 H5 *ehxA* and *eaeA*
5 GB10 *ehxA*
6 GB10 *ehxA*
7 GB10 *ehxA*
8 H5 *ehxA* and *eaeA*
9 GB5 *stx*-2
10 H5 *ehxA* and *eaeA*
11 H10 *stx-2* and *eaeA*
12 H5 *ehxA* and *eaeA*

Figure 15 Pulsed field gel electrophoresis analysis of strains of *E. coli* isolated from the hide and ground beef from animals 5 and 10. Strains are grouped according to their percent relatedness (Dice Coefficient). All strains are listed by sample type and virulence profile. H, hide. GH, ground beef. ET, Electrophoretic type.

stx-2 gene. The final ET contained two strains that were less than 70% related. One strain was isolated from the hide of animal 10 and the other from the hide of animal 5. The strain isolated from animal 10 had possessed the *stx*-2 and *ehxA* genes, whereas the strain from animal 5 had the *ehxA* and the *eaeA* genes. These results indicate strains with different virulence genes can be closely related, whereas strains with the same genes can also be distantly related (Figure 15).

4.9 Identification of EHEC virulence genes in faecal, hide, caecal, and ground beef enrichment broths obtained throughout the beef cattle production continuum:

To identify the EHEC virulence genes (*ehxA*, *stx*-1, *stx*-2, and *eaeA*) present in enrichment broths prepared from samples obtained at different points in the production continuum, total genomic DNA was purified from the enrichment broths of faecal (1 and 5 months on pasture; 1 and 4 months in feedlot), caecal, hide and ground beef (from first and second slaughter days) samples. One hundred and ninty three of the 210 enrichment broths tested by PCR were positive for the presence of the *uspA* gene. All samples that were positive for the *uspA* gene were further characterized by PCR using the four virulence genes described previously. Only DNA from enrichment broths with multiple virulence factors, which may indicate the presence of *E. coli* O157:H7 were tested for the presence of the non-functional *uidA* gene. For ease of analysis, samples were separated into two groups of 15 animals by their slaughter dates (March 21st and April 3rd).

Table 13 shows an example of the PCR results that were obtained for faecal enrichment broths from the first 15 animals after 1 month on pasture. The data for all other PCR analysis can be found in Appendix C. Virulence profiles (VP), which use numbers that are assigned to signify a specific combination of virulence genes in a

Table 13: EHEC virulence factors and marker genes detected in purified genomic DNA from the faecal samples obtained from cattle after one month on pasture and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*	
1	+	-	+	-	-	nd[c]	11
2	+	+	-	-	-	nd	3
3	+	-	-	+	-	nd	6
4	+	-	+	-	-	nd	11
5	+	+	+	-	+	nd	9
6	+	+	+	+	-	-	5
7	+	+	+	-	-	nd	10
8	+	+	+	+	+	-	1
9	+	-	-	+	-	nd	6
10	+	-	-	-	-	nd	0
11	+	-	-	+	-	nd	6
12	+	+	+	+	-	+	5
13	+	+	-	+	-	nd	2
14	+	-	-	+	-	nd	6
15	+	+	-	+	-	-	2

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

sample, were assigned to each enrichment broth that was analyzed. Table 14 shows all possible combination of virulence genes and a corresponding VP.

The VP of enrichment broths obtained from animals slaughtered on March 21st, are listed in Table 15 and the VP for enrichment broths from animals slaughtered on April 3rd are summarized in Table 16. For the 30 animals in this study there was a total of 15 VP detected. For the enrichment broths obtained on the first slaughter date (March 21st) the most common virulence gene was the *ehxA*, which was found in 46.6% of all the samples tested. The *stx*-1, *stx*-2 and *eaeA* genes were found in 31.4, 33.3 and 29.5% of samples respectively. The percentage of enrichment broths that tested positive for the virulence genes was higher for samples from the first slaughter date compared to the percentage of enrichment broths collected from animals second slaughter date. However, the *stx*-2 gene was detected in the same percentage of enrichment broths, regardless of the slaughter date (Figure 16).

Overall, 35.2% of samples were negative for all virulence factors. There were 17 samples that were negative for the *uspA* gene, one from the hide of animal 28 and 16 from enriched broths of ground beef samples. A total of 35 of 210 enrichment broths were tested for the presence of the *E coli* O157:H7 *uidA* gene, and the gene was detected in 11 of these enrichment broths. Of the 11 enrichment broths that were positive for the presence of the *E. coli* O157:H7 *uidA*, three were from enrichment broths from faecal samples obtained from animals after one month on pasture, three were from enrichment broths from faecal samples obtained from animals after four months in feedlot and five were from enrichment broths from hide samples obtained from the hides of animals at the first slaughter.

Table 14: Virulence profiles used for classification of EHEC virulence traits virulence genes detected in faecal, hide, caecal and ground beef enrichment broths obtained from beef cattle at the Lacombe Research Centre.

Virulence	Virulence Genes			
Profile	*stx*-1	*stx*-2	*eaeA*	*ehxA*
0[a]	-	-	-	-
1	+	+	+	+
2	-	+	-	+
3	-	-	-	+
4	+	+	+	-
5	+	+	-	+
6	-	+	-	-
7	-	-	+	-
8	-	-	+	+
9	+	-	+	+
10	+	-	-	+
11	+	-	-	-
12	+	-	+	-
13	-	+	+	+
14	+	+	-	-
15	-	+	+	-

[a] Virulence profiles assigned for combinations of virulence genes.

Table 15: Virulence profiles determined by PCR analysis of DNA purified from faecal samples obtained on two occasions while cattle were on pasture and in the feedlot, and hide, caecal and ground beef samples enriched in mTSB-n. Cattle were slaughtered on March 21st, 2000.

	Pasture[a]		Feedlot[a]		Hide[b]	Ceacal[c]	Ground Beef[d]
Animal #	July 5th	October 20th	November 20th	February 14th	March 21st	March 21st	March 24th
1	11[d]	3	6	0	15	0	0
2	3	0	3	9	1	11	0
3	6	3	0	12	8	1	0
4	11	0	6	11	8	0	8
5	8	10	0	7	1	8	8
6	5	11	5	6	8	0	0
7	10	3	11	6	1	3	0
8	1	0	0	0	8	1	8
9	6	10	0	6	4	2	0
10	0	3	0	3	4	10	8
11	6	3	0	0	5	1	0
12	5	0	11	2	1	8	6
13	2	0	0	6	1	8	6
14	6	11	3	10	1	3	0
15	2	3	3	13	1	8	11

[a] 25 g faecal grab sample.

[b] 1000 cm^2 sponge swab sample.

[c] 25 g faecal sample from ceacum after slaughter.

[d] 25 g composite sample of ground beef.

[d] Virulence profile using selected virulence genes *stx*-1, *stx*-2, *eaeA* and *ehxA*.

Table 16: Virulence profiles determined by PCR analysis of DNA purified from faecal samples obtained on two occasions while cattle were on pasture and in the feedlot, and hide, caecal and ground beef samples enriched in mTSB-n. Cattle were slaughtered on April 3rd, 2000.

	Pasture[a]		Feedlot[a]		Hide[b]	Ceacal[c]	Ground Beef[d]
Animal #	July 5th	October 20th	November 20th	February 14th	April 3rd	April 3rd	April 6th
16	14[d]	3	6	5	6	11	0
17	4	3	0	6	6	0	0
18	2	3	6	1	6	3	0
19	6	3	6	2	6	6	0
20	0	0	3	0	6	0	0
21	12	3	0	0	6	0	0
22	0	0	6	0	0	0	0
23	0	0	6	0	6	6	0
24	2	3	0	0	0	0	0
25	3	10	10	10	0	8	0
26	11	0	6	1	3	3	0
27	14	11	10	14	6	2	0
28	15	0	0	6	0	0	0
29	6	3	0	5	0	0	0
30	6	10	0	0	6	8	0

[a] 25 g faecal grab sample.

[b] 1000 cm^2 sponge swab sample.

[c] 25 g faecal sample from ceacum after slaughter.

[d] 25 g composite sample of ground beef.

[d] Virulence Profile using selected virulence genes *stx*-2, *stx*-1, *eaeA* and *ehxA*.

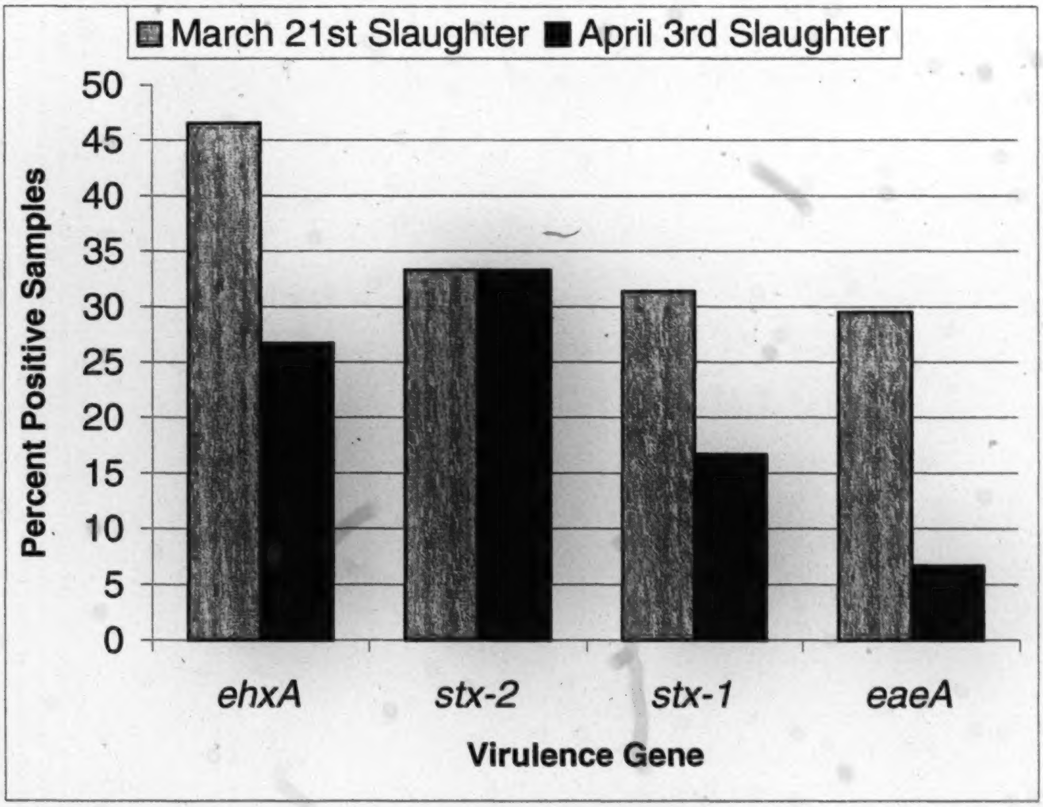


Figure 16: Percentage of the faecal, hide, caecal and ground beef samples collected from animals slaughtered on March 21st or April 3rd, 2000 that tested positive for the four selected virulence genes by PCR.

Figures 17 and 18 shows the percentage of samples obtained at each sampling time for animals slaughtered on March 21st and April 3rd, respectively, that were positive for the presence of each of the four virulence genes as determined by PCR analysis. Each of the virulence genes were detected in samples from animals slaughtered on March 21 (Figure 17) with the exception of the *stx*-2 gene, which was not detected in the second group of samples obtained while animals were on pasture and the *eaeA* gene, which was not detected in the first samples taken while animals were in the feedlot. The greatest percentage of samples that tested positive for virulence genes were obtained from the hides of the animals.

Samples obtained from the hide, caecum and ground beef of animals slaughtered on March 21st (Figure 17) had a higher percentage of samples that were positive for the presence of the virulence genes than samples obtained from animals that were slaughtered on April 3rd (Figure 18). Samples from animals slaughtered on April 3rd tended to have fewer samples that were positive for the presence of all of the four different virulence genes. Virulence genes were not detected in any of the ground beef samples obtained from the 1[illegible] cattle slaughtered on April 3rd.

Enrichment broths from hide samples from animals 1 to 10 were plated on SD-39 and TBX agar to determine the selectivity of VCCWBA. A total of 96 haemolytic colonies were picked from VCCWBA and all were green and blue on SD-39 and TBX agar, respectively. This indicated that all strains picked from the VCCWBA plates were E. coli. All of these strains also tested positive for the presence of the uspA gene by PCR. None of the 96 colonies were positive for the ehxA gene when tested by PCR.

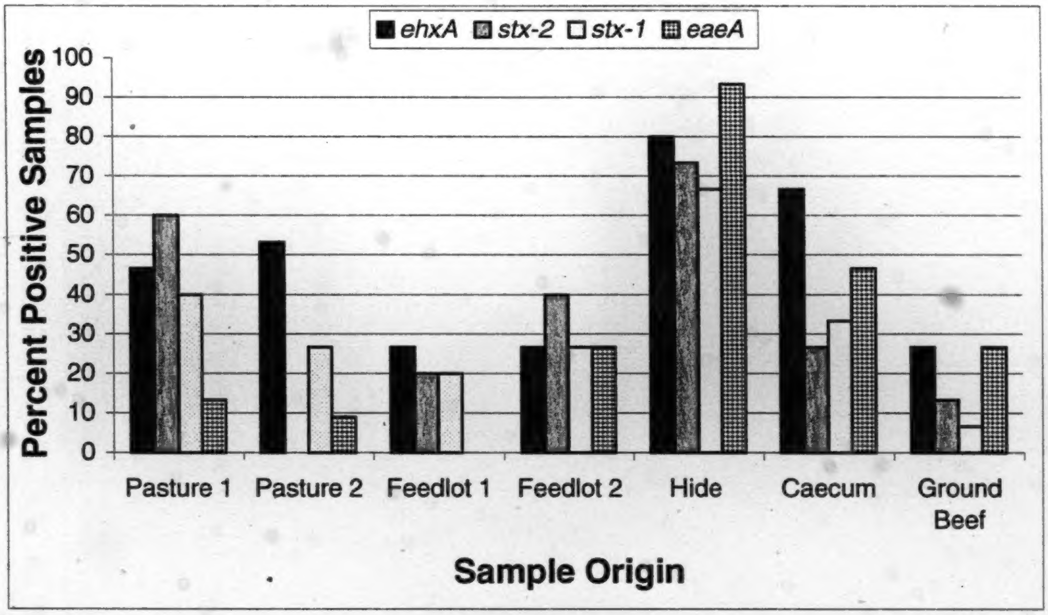


Figure 17: Percentage of samples obtained from the group of cattle slaughtered on March 21st, 2000 that PCR analysis detected the presence of each of the four EHEC virulence genes, *ehxA, stx*-1, *stx*-2 and *eaeA*.

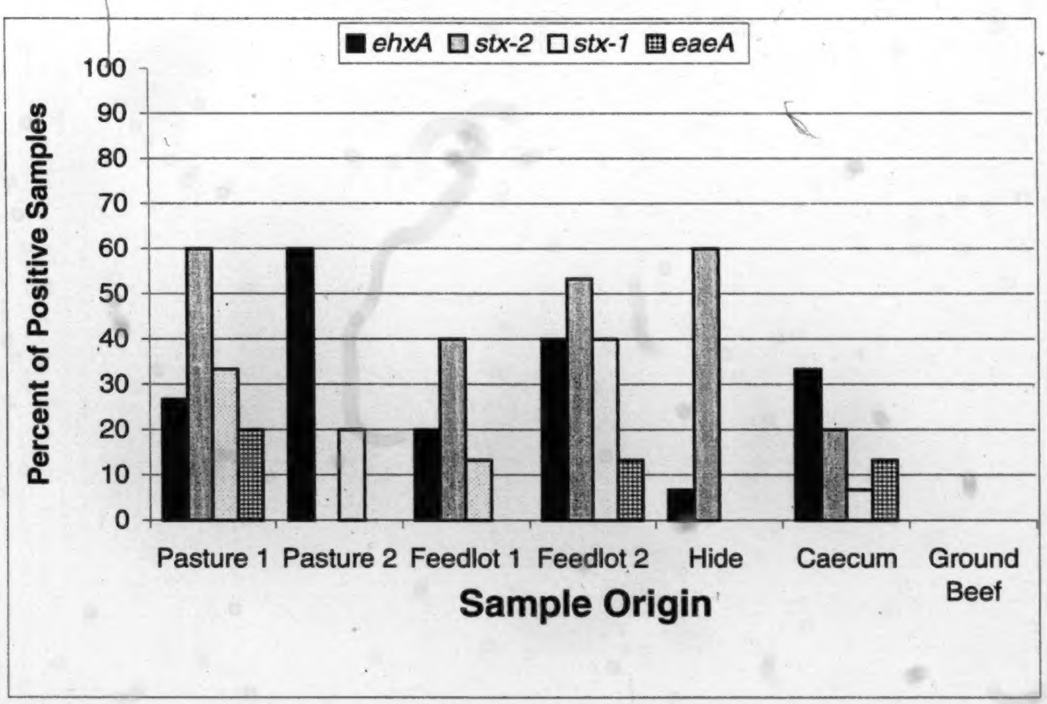


Figure 18: Percentage of samples obtained from the group of cattle slaughtered on April 3rd, 2000 that PCR analysis detected the presence of each of the four EHEC virulence genes, *ehxA*, *stx*-1, *stx*-2 and *eaeA*.

5 Discussion and Conclusion

5.1 Isolation and characterization of complex STEC strains:

There is no direct information that demonstrates how EHEC contaminates beef products, although the obvious route is via carcass surface contamination during processing. During slaughter, hide and faecal material are potential sources of contamination. Although there is very little known about STEC on cattle hides (Arthur et al., 2002), a recent report by Elder et al. (2000) reported that *E. coli* O157:H7 frequently contaminates cattle hides. If *E. coli* O157:H7 is frequently found on cattle hides, there is the possibility that non-O157 STEC could also be present on hides. Contamination of an animal's hide can occur from a number of sources, including soil and faeces.(Bell 1997; Brown et al., 2000).

E. coli O157:H7 has some features (inability to ferment sorbitol, and the lack of β-glucuronidase activity) that allow differentiation of this group of strains from other strains of *E. coli*. In comparison, the isolation of non-O157:H7 STEC strains is a difficult procedure due to the lack of a conserved phenotypes amongst these strains that permits consistent isolation of all strains (Arthur et al., 2002). The use of VCCWBA has been suggested by Lehmacher et al., (1998) as a good method for the isolation of complex STEC (*stx* and accessory virulence genes present in one strain). These complex STEC strains are more likely to cause human disease than STEC strains without any accessory virulence genes (Hornitzky et al., 2001).

In this study, samples obtained from the hide and caecum from 30 head of cattle were enriched in mTSB-n and screened for the presence of *stx*-2 and *ehxA* genes using PCR. The *ehxA* gene was used because it has been suggested as a good virulence marker for STEC (Lehmacher et al., 1998; Schmidt et al., 1995). The *stx*-2 gene was also chosen because it is more often associated with serious illnesses in humans than *stx*-1 (Nataro and Kaper 1998; Ostroff et al., 1989). PCR analysis detected the presence of the *ehxA* DNA sequence in 60% (19/30) of the enrichment broths prepared from samples taken from the hide and 50% (15/30) of the enrichment broths prepared from the ceacal samples. The *stx*-2 gene was detected in 36.6% (11/30) of the samples from the hide and ceacum. To the author's knowledge, there are no reports in the research literature on the frequency of the presence of either the *ehxA* or *stx*-2 genes in non-O157:H7 *E. coli* strains isolated from cattle hides. Hornitzky et al. (2001) reported that PCR could detect the presence of the *ehxA* and *stx*-2 genes in 59.3% (73/123) and 30.1% (37/123), respectively, of enrichment broths prepared from the faecal contents obtained from beef cattle. Although the current study did not have the same number of faecal isolates as the studies mentioned above, the frequency of detection of the DNA sequences for *ehxA* and *stx*-2 genes in enrichment broths was similar.

Ceacal samples were analyzed for the presence of the genes from the virulence factors of EHEC because this gives a good indication of the intestinal carriage of potentially pathogenic *E. coli* by animals at the time of slaughter. These data can be compared to data for samples collected from the hides to give an indication of the relatedness of strains of *E. coli* from the environment and from intestinal carriage.

Detection of the DNA from the *ehxA* and *stx*-2 genes show that most of the samples from the hide and ceacum were positive for the presence of these genes; however, there were a few differences. In a few cases, the samples from the hide were positive while the ceacal samples were negative. The only exception to this was the data for animal 30, where the ceacal sample was positive and the hide sample was negative for the presence of *ehxA* gene. These data indicate that each animal had contamination on the hide that may have originated from its own intestinal contents or from that of other animals in the same pen. The results of this study also showed that cross-contamination among animals within a feedlot pen might be important in the ecology of potentially pathogenic *E. coli*. The first 15 animals that were slaughtered had a much higher percentage of samples that were positive for potentially pathogenic *E. coli* than samples from animals that were housed in a separate, although adjacent pen and slaughtered as the second group of cattle. This supports the suggestion by Midgley and Desmarchelier (2001) that identification of animals that carry potentially pathogenic *E. coli* and segregation of these animals could be a useful management practice to reduce the incidence of potentially pathogenic *E. coli* in meat.

Samples from the three animals that were positive for the presence of DNA from the *ehxA* and *stx*-2 genes in the initial screening of enrichment broths were chosen for further characterization. Strains of *E. coli* that were enterohaemolytic on VCCWBA were isolated and these specific strains were subjected to profiling of virulence genes. In addition, to confirm that samples that were negative in initial screening did not result in the isolation of strains of *E. coli* with virulence genes, enrichment broths from the faecal, hide, and ground beef samples of an animal, which was negative for the *ehxA* and *stx*-2 genes during preliminary screening, were plated onto VCCWBA. No colonies with small turbid zones of haemolysis were isolated from this sample. This indicates that the initial PCR analysis of the enrichment broths was a useful tool for preliminary screening of samples.

In the current study, the VCCWBA plates were incubated anaerobically for 30 h, which is unlike the aerobic incubation for 20 to 24 h used by Lehmacher et al., (1998). This change in procedure was made for the current study as preliminary work indicated that no haemolysis was observed on plates inoculated with *E. coli* O157:H7 (ATCC 43895) after 20 to 24 h of aerobic incubation. Preliminary work showed that anaerobic incubation of VCCWBA resulted in a large number of colonies with small turbid zones of haemolysis with reference stains of *E. coli* O157:H7 (ATCC 43895), and that reference strains of *E. coli* biotype 1 (ATCC 25922) did not exhibit any haemolysis which was as expected. There were a number of strains from the current study that had small turbid zones of haemolysis on VCCWBA plates but were negative for the presence of *ehxA* using PCR. This could have been due to the inhibition of PCR because the DNA used was isolated by boiling. It has been reported that many media components in minute quantities can inhibit PCR (Rossen et al., 1992; Abolmaaty et al., 1998). However, this is not likely because a selection of isolates were also analyzed for the presence of virulence genes using DNA purified with a DNA purification kit and they had the same PCR profiles as strains that were analyzed using DNA isolated by boiling.

In this study, 38.5% (75/195) of *E. coli* colonies picked from VCCWBA plates possessed the *ehxA* gene. This is low compared to a study by Djordjevic et al. (2001) that found that 89.3% of strains with small turbid zones of haemolysis were positive for *ehxA* using PCR. It has been reported by a number of authors that 72 to 93% of strains which have the gene also possess *stx*-1 or *stx*-2 genes (Bettelheim 1995; Kobayashi et al., 2001 and Hornitzky et al., 2001). Sixteen of 75 (21%) strains isolated in the current study that were positive for *ehxA* were also positive for *stx*-1 or *stx*-2. This is lower than the percentage reported in other studies, which showed a high association between the presence of the *ehxA* gene and one or both of the *stx* genes (Bettelheim 1995; Kobayashi et al., 2001). Thirty-two of 75 strains (43%) isolated in the current study had the *eaeA* gene, which was higher than the 16.3% (Djodjevic et al., 2001) and 23.9% (Kobayashi et al., 2001) that has previously been reported for strains isolated from cattle.

In general, samples from the hides of the animals had the greatest number of strains (56/75) with virulence genes, whereas ground beef samples had the fewest number of strains (4/75) with virulence genes. Of the total number of strains with virulence genes that were isolated from the samples obtained from these animals, only 21.3% (17/75) of the strains could be classified as STEC (based on the presence of one or both *stx* genes) and the majority of these strains were isolated from enrichment broths prepared from samples from the hides. The virulence patterns of the strains that were isolated from the faecal samples and tentatively identified as STEC did not match any of virulence profiles of the STEC strains isolated from the hide samples. This could indicate that contamination with STEC was a result of contamination among cattle in the feedlot or that carriage of STEC changes with time. A number of researchers have reported that faecal carriage of strains of STEC can change over time (Zhao et al., 1995; Faith et al., 1996; Hancock et al., 1997). It was not possible to determine the virulence profiles of strains isolated from ceacal samples due to difficulties experienced with PCR analysis of these samples. This could be due to a number of differences in the methods used in this study as compared to those used in previous studies.

Ground beef samples had the fewest number of isolates that exhibited haemolysis on VCCWBA, and thus the failure to isolate potentially pathogenic *E. coli* strains was not a surprise. However, the low number of potentially pathogenic *E. coli* isolated from the faecal samples was somewhat surprising. This may have been a result of the presence of a high number of background microorganisms present in faecal samples, which could have masked the small number of colonies that would produce haemolysis on the VCCWBA plates (Bettelheim 1995; Hornitzky et al., 2001). This may in part also account for the isolation of greater numbers of potentially pathogenic *E. coli* from the hide samples, as the anaerobic incubation of the VCCWBA plates would have inhibited the competition of the microflora found on the hide, which would likely be composed of a high percentage of aerobic microorganisms. The hight temperature of incubation (37°C) may have also limited growth of non-enteric bacteria present on the hide. The majority of faecal samples were collected in the fall and winter when the prevalence of *E. coli* O157:H7 has been reported to be low (Hancock et al., 1997). The samples collected at slaughter were collected in March and April, and had a higher incidence of potentially pathogenic *E. coli* compared to the other samples collected in this study. This period of time has been associated with increased shedding of *E. coli* O157:H7 (Hancock et al., 1997). Further research must be conducted but these preliminary data may indicate that non-O157 STEC have periodic shedding that increases in warmer months like *E. coli* O157:H7.

One of the drawbacks of determining the presence of the *ehxA* gene in *E. coli* is that it is carried on a plasmid that could be lost during subculturing of strains. A number of studies have reported cases where cultures first test positive for *ehxA* and then become negative upon sub-culturing (Beutin et al., 1989; Scotland et al., 1990; Schmidt et al., 1994). This is of concern in this study because frozen cultures were revived in enrichment broth and then plated on VCCWBA. The culturing procedure may have resulted in the loss of the plasmid carrying the *ehxA* genes thus the data from this study may underestimate the prevalence of *E. coli* that carry the *ehxA* gene. Vancomycin-cefixime-cefsulodin washed sheep blood agar plates are able to isolate *E. coli* strains that possess genetic variants of the *ehxA* genes (Lehmacher et al., 1998) that may not test positive with the PCR procedure used in this study.

One of the limitations of VCCWBA as a selective medium is that it may not select for strains of STEC with the *eaeA* gene that do not carry the *ehxA* gene. Methodologies need to be developed to facilitate the rapid isolation of these strains of STEC.

The low numbers of STEC isolated from the faecal samples analyzed in this study could also be a result of the use of mTSB-n as an enrichment broth. It is possible that the use of mTSB-n resulted in the selective enrichment of a subgroup of strains of STEC. The combination of growth in mTSB-n with selective plating onto VCCWBA could have underestimated the number of different strains of STEC due to the inhibition of the growth of sublethally damaged cells. To recover sublethally injured cells, other researchers have used incubation in a non-selective enrichment broth for a short time (4 to 6 h) prior to incubation in selective media (Chapman et al., 2001; Uyttendaele et al., 2001). However, this procedure would not allow for the selective isolation of strains of STEC and the results may be biased by the overgrowth of non-STEC stains.

Direct plating of samples could have been done to eliminate the underestimation of the prevalence of some strains; however the low numbers of STEC commonly present in samples would be overgrown by the competitive microflora. This was demonstrated by Hornitzky et al. (2001) who compared results obtained by direct plating of faecal samples on VCCWBA, with those obtained by plating enrichment broths of the same faecal samples. They found a greater number of STEC strains with accessory virulence genes from the faecal samples that had been enriched in modified *E. coli* broth.

In the current study, the most common strains isolated on VCCWBA were those that possessed the *ehxA* (38 strains); the *ehxA* and *eaeA* (18 strains); and the *ehxA, stx*-1, *stx*-2 and *eaeA* (11 stains) virulence genes. In the study by Hornitzky et al. (2001), the most common virulence genes detected in strains isolated on VCCWBA had *ehxA* (nine strains), *eaeA* and *stx*-1 (eight strains); *ehxA* ; *ehxA* and *stx*-2 (seven stains); *ehxA* and *eaeA* (six strains). In both studies there were a number of STEC strains isolated that had the virulence genes, that would potentially allow these strains to cause serious illnesses in humans.

It is difficult to compare the results of the current study to that of others, because almost all researchers use the O and H serotype for the identification of *E. coli* strains present in enriched samples. Traditionally, serotype was the only method available to fingerprint microorganisms. However, a newer method that has been described is virotyping, which categorizes microorganisms by their virulence traits using their virulence profile determined using PCR or DNA hybridization procedures (Nataro and Kaper 1998, Salyers et al., 2002). Serotyping is not really necessary when examining the potential virulence of strains because any serotype has the ability to cause illness if the appropriate virulence traits are present (Salyers et al., 2002). This has been demonstrated by *E. coli* O157:H7 strains that have no virulence traits and cannot cause illness whereas others are known pathogens that can cause illness with as few as 10 CFU (Armstrong et al., 1996; Nataro and Kaper 1998). Even if the serotype is known, genetic analysis will still need to be performed to determine the potential pathogenicity of *E. coli*. Serotyping was not done on the strains isolated from the samples collected in this study. It is possible that the strains with the same VP isolated from different samples are actually the same strain and thus could be over represented in the data set.

E. coli strains from a culture collection as well as those isolated in the current study where characterized by PCR and RDBH. This was done to aid in the development of the RDBH and to ensure the PCR procedure used was reliable. These two procedures gave comparable results, with the exception of a discrepancy in the detection of *stx*-1 and *stx*-2 genes. Sequence analysis of the PCR amplified products in this study for all virulence genes were highly homologous (98 to 100%) with published sequences, thus the PCR was accurate, whereas the RDBH procedure was not able to detect all positive strains. Upon further study, it was found that the *stx*-1 and *stx*-2 genes were not being correctly amplified in the RDBH multiplex PCR. With some slight modifications the RDBH procedure will provide a quick isolation and characterization procedure for non-O157:H7 strians of STEC.

Colony and slot-blot hybridizations were used to compare methods available for the isolation of non-O157:H7 STEC. Reliable results could not be obtained with colony blot hybridization. This may have been due to the selection of the *uspA* and *ehxA* genes as probes. The amplification products for *uspA* have high homologies with stress proteins from other members of the *Enterobacteriaceae*, including *Y. enterocolitica*. The *ehxA* probe used is encoded on a plasmid, which may have caused some problems with the colony hybridization procedure, due to the loss of the plasmid, or low plasmid copy numbers (Schmidt et al., 1994; Beutin et al., 1989). Slot-blot hybridization was the only method that yielded similar results to that achieved with PCR analysis. Slot-blot hybridization was time consuming, requiring DNA purification, along with probing and processing time required for the slot-blot hybridization procedure, and it requires the use of radioactive material. Compared to these two procedures, characterization of STEC isolates using VCCWBA and PCR provided the best combination of ease of procedure and time required for analysis, although this method also has many pitfalls. There is still a need for the development of a method or combination of methods that allow for consistent isolation of all complex STEC strains.

Eleven strains isolated from the enrichment broths prepared from the hide samples were positive for the *uidA* gene. Random amplification of polymorphic DNA analysis and PFGE was done on these 11 possible *E. coli* O157:H7 strains, and there were no distinguishable differences among any of the strains using either method. This indicates that all 11 isolates are clonal. These 11 strains also possessed the same *fliC* pattern determined by PCR-RFLP, which was identical to previously published profiles for *E. coli* O157:H7 (Fields et al., 1997), indicating the presence of the H7 flagellar antigen. These 11 colonies also exhibited classical phenotypes indicative of *E. coli* O157:H7 on CT-SMAC and SD-39 agar. The fact that *E. coli* O157:H7 was isolated from the hides and not faecal or ground beef samples is not a surprise, as faecal prevalence of *E. coli* O157:H7 has been reported to range from 5 to 37% (Chapman et al., 1997a) with the highest prevalence in the summer, whereas the prevalence on ground beef has been reported to be between 0 and 4% (Doyle and Schonei, 1987; Samadopour et al., 1994; Acheson et al., 1996). Prevalence on hides has been reported to be around

18 to 23% (Ransom et al., 2002), whereas in the current study one out of three hides tested positive for *E. coli* O157:H7, although with this small sample size no significant correlation can be made.

From each faecal, hide and ground beef sample for each of the animals tested, there were strains isolated that had both the *uspA* and *ehxA* genes. This demonstrates the possibility that the same strains of potentially pathogenic *E. coli* are being transferred from faeces through to the ground beef during the slaughter and cutting processes. However, when each isolate was analyzed using PFGE, strains with the same virulence traits from one sample were related, but those from different animals or sample times were not. This does not conclusively prove that related strains were not transferred through the production process. It is possible that strains that were related to those that were isolated from the samples were missed due to problems with recovery methods. Although the use of PFGE is currently the best technique available to determine strain relatedness, it is important to note that PFGE banding patterns can change with each culture as a result of the gain or loss of mobile genetic elements (Barkocy-Gallagher et al., 2001).

5.2 Characterization of EHEC virulence traits through the cattle production continuum:

There have been very few studies done that examine the presence of virulence genes from EHEC throughout the entire cattle production continuum, especially with the ability to track specific animals through the entire process. The facilities at the Lacombe Research Centre allowed the ability to track 30 head of cattle from pasture to feedlot and through the slaughter process. Elder et al. (2000) followed *E. coli* O157:H7 from faeces, hides and carcasses in a cattle processing plant, but they could not directly link isolates from specific animals throughout the process. This study also only considered *E. coli* O157:H7 and not non-O157 STEC.

In the current study, there were a total of 210 samples examined. There were 120 faecal, 30 hide, 30 caecal and 30 ground beef samples that were enriched in mTSB-n and characterized by PCR. One hundred and ninety-three (91.9%) of the samples tested positive for *E. coli*. Of the 17 samples that were negative for *E. coli*, 15 were from ground beef and one was from a hide. The one hide sample that tested negative for the presence of *E. coli* also tested negative for all EHEC virulence genes. The high percentage (53.3%) of negative samples from ground beef was probably due to the small number of animals (15) processed at the research abattoir on each of the two slaughter dates. The Lacombe Research Centre is an extremely clean facility that employs highly skilled workers who follow a well developed HACCP program.

Enrichment broths that did not test positive for the presence of *E. coli* were not included in the PCR analysis for virulence genes. Table 17 summarizes the percentages of strains isolated at the different sampling times with each of the virulence genes and compares the results of the current study to those of Hornitzky et al. (2001) and Djordjevic et al. (2001). In the current study, the *ehxA* gene was the most common virulence gene detected. Results from Hornitsky et al. (2001) also reported that the *ehxA* gene was the most prevalent virulence gene detected in faecal samples from cattle at the time of slaughter; however, Djordjevic et al (2001) reported that the *stx*-1 gene was the most common virulence gene in strains of *E. coli* isolated from the faeces of sheep at the time of slaughter. In all three studies, the *eaeA* gene was isolated less frequently than any of the other virulence genes. Strains of STEC isolated from animals have a higher percentage of *stx*-1 genes than *stx*-2 genes when compared to STEC strains isolated from humans (Gyles et al., 1998; Boerlin et al., 1999). The higher prevalence of the *stx*-1 genes in samples obtained from animals was not confirmed in the present study as the prevalence of *stx*-1 and *stx*-2 genes varied depending on the sample and usually, the prevalence of *stx*-2 genes was higher. The difference may be due to the fact that other researchers only examined the presence of EHEC virulence traits in faecal samples and did not evaluate hide, caecal or ground beef samples (Gyles et al., 1998; Boerlin et al., 1999). In addition, these studies were only done on samples obtained at one sampling time. This makes it difficult to compare results since it is known that the shedding of STEC can vary with time (Besser et al., 1997; Wells et al., 1991).

Table 17: Percentage of samples from cattle and sheep positive for the EHEC virulence genes *ehxA, stx*-2, *stx*-1, and *eaeA*.

Virulence genes	Cattle throughout the production continuum[a] (n=193)	Cattle faeces from pasture[b] (n=60)	Cattle faeces from feedlot[c] (n=60)	Cattle faeces from, pasture, feedlot and caecum[d] (n=30)	Cattle faeces at slaughter[e] (n=123)	Sheep faeces at slaughter[f] (n=904)
ehxA	40.9	51.7	33.0	44.7	57.7	38.3
stx 2	36.8	30.0	40.0	32.0	30.1	37.8
stx-1	25.9	30.0	25.0	26.0	49.6	72.0
eaeA	19.2	8.3	8.3	12.5	22.0	10.4

[a] All samples, including hide and ground beef from this study.
[b] From this study.
[c] From this study.
[d] All faecal and caecal samples used in this study
[e] Hornitzky et al. (2001).
[f] Djordjevic et al. (2001)

The presence of virulence genes at each sampling time from pasture grazing, feedlot and slaughter (hide, caecal and ground beef) varies with time. The only consistent trend is that the samples from animals killed on the first slaughter date had a higher presence of virulence genes than samples from those killed on the second slaughter date. The only difference for this group of animals while they were in the feedlot, was that they were separated into adjacent pens with a shared water supply. Midgley and Desmarchelier (2000) reported that contaminated soils in feedlot pens could contribute to the transmission cycle of non-O157 STEC strains. The data in this study shows that there were slight differences in the detection of virulence genes in the samples from animals in the separate pens. The greatest difference was observed in the percentage of samples that were positive for the presence of the *stx*-2 and *eaeA* genes. The small sample size made statistical analysis of this data inappropriate. These results may suggest that the different feedlot pens contributed to the presence of different STEC virulence genes detected in the EHEC from the faeces and hide samples of the cattle.

Animals from the separate pens were also slaughtered on two slaughter dates (March 21st and April 3rd, 2001). The EHEC virulence genes detected in the hide samples obtained from the two groups of animals was different. For the first slaughter group (animals 1 to 15), 53.3, 36.6, 36.6, and 24.4% of the hide samples were positive for the *ehxA*, *stx*-2, *stx*-1 and *eaeA* genes, respectively. These numbers are close to those reported by Hornitzky et al. (2001), with the exception of the lower percentage of samples positive for the *stx*-1 gene in this study. The second slaughter group (animals 16 to 30) 30.0, 38.9, 18.9 and 10% of hide samples were positive for the *ehxA*, *stx*-2, *stx*-1, and *eaeA* genes, respectively. These numbers are closer to those reported by Djordjevic et al. (2001), again with the exception of the lower percentage of samples positive for the *stx*-1 gene. These results show the difference that can be detected in the presence of

STEC virulence traits over time, and support the previous suggestions that STEC virulence genes can fluctuate with time.

The only difference between the animals, apart from them being held in adjacent pens in the feedlot, was that only steers were slaughtered on March 21st and heifers were slaughtered on April 3rd. Although this study was not set up to determine the difference in carriage of STEC strains in steers or heifers it has been reported by Van Donkersgoed et al. (1999) that 62% of faecal samples obtained from male cattle and 38% of faecal samples obtained from female cattle were positive for the presence of the *E. coli stx* genes. This was in contrast to the results of a study by Wells et al. (1991) that reported that heifers had a greater rate of faecal shedding of strains of STEC. In this study there were a greater number of hide and caecal samples that were positive for EHEC virulence genes from animals slaughtered on the first day than the second day. These results suggest that further studies should be done to determine if there is a true difference in the prevalence of STEC in steers and heifers.

Twenty-six of 30 faecal samples from animals after one month on pasture were positive for at least one virulence gene, with a total of 13 different VP detected. This was the highest number of samples from animals and greatest number of VP detected for any sample time. This is in agreement with Wells et al. (1991) who also reported that the level of secretion of STEC in faeces is highest in calves just after weaning. The cattle in this study were placed onto pasture post weaning. Midgley et al. (1999) reported that there is a considerable amount of variation in the detection of STEC virulence genes when sampling is done repeatedly over time. There were a total of 15 VP detected in this study. A minimum of one sample from each animal in this study was positive for the presence of *E. coli* with at least one virulence gene. The virulence profiles of the samples changed as animals moved through the production continuum, which reinforces the need for samples to be taken a number of times to get an accurate assessment of the carriage of STEC strains by cattle.

There have been numerous studies reporting the low level of *E. coli* which carry the *eaeA* gene in samples from the bovine environment (Gyles et al., 1998; Boerlin et al., 1999; Bertin et al., 2001; Arthur et al., 2002). In this study, the *eaeA* gene was the gene that was detected least often in all samples. The low number of samples that tested positive for the *eaeA* gene would seem to indicate that the presence of *E. coli* that have the ability to cause serious illness was very low. However, a number of factors including the fact that samples in this study were collected from a research cattle herd make it difficult to generalize about the carriage of EHEC in cattle populations. Boerlin et al. (1999) reported that 42.5% of STEC strains isolated from stool samples from humans were positive for the *eaeA* gene and only 23.5% of faecal samples from cattle were positive for the *eaeA* gene. This suggests that STEC strains have evolved to inhabit different environments and required different genetic mechanisms to survive in these environments. This is supported by the observation of Beutin et al. (1995) that many STEC from healthy animals are limited in their host range and most strains may not be able to colonize animals outside a specific host range.

In the current study, PCR of the *uidA* gene was done to detect the presence of the non-functional GUD gene from *E. coli* O157:H7 in faecal and hide samples from cattle. Eleven samples tested positive for the presence of DNA from this gene. This indicates that *E. coli* O157:H7 had a low prevalence in this cattle population. In the first portion of this study, *E. coli* O157:H7 was isolated from the hide sample of animal 10 using VCCWBA plates. However, the same sample did not test positive for the *uidA* gene when testing hide samples enriched in mTSB-n in the second portion of this study. This may be due to the reportedly low incidence of *E. coli* O157:H7 in the large population of competitive organisms that are found in bovine samples (Nataro and Kaper, 1998). This may also be a result of an enrichment broth that was frozen and then subcultured into mTSB-n for regrowth and analysis by PCR. The comparison of the two sample times is difficult because the possibility of a different microbial composition of the portion of sample analyzed in each study. This emphasizes the fact that the low overall representation of STEC, compared to the rest of the bacterial microflora in bovine samples, can give varying results depending on what portion of the sample is tested.

In conclusion, virulence genes from EHEC are commonly found in beef cattle at different stages throughout the production continuum. Although many of the bovine samples lack the accessory virulence genes thought to be necessary to cause disease in humans, many samples have been found that have *E. coli* with the same VP as strains that have been linked to human disease. Until evidence is obtained to distinguish between pathogenic and non-pathogenic STEC, all STEC will have to be treated as potential sources of foodborne illnesses (Gyles et al., 1998).

If enrichment broths favour the growth of specific strains of *E. coli*, then it may be necessary to use both primary culture and an enrichment step to increase the likelihood of isolating complex STEC. Although methods such as colony hybridization in association with hydrophobic grid membrane filtration may improve the recovery rates of less prevalent populations of potentially virulent *E. coli* (eg. STEC strains containing the *eaeA* gene), these approaches are labour intensive, time consuming, and logistically may not be easily accommodated in future studies (Djordjevic et al., 2001).

Strains of *E. coli* with specific virulence genes were isolated from faecal, hide and ground beef samples. However, there was no direct evidence from PFGE or RAPD that genetically linked the strains from different samples. The lack of the detection of any genetic link between the strains isolated may have been due to the small number of strains examined for each sample and thus, conclusions cannot be made about prevalence of the different organisms in the samples. However, the current study has demonstrated that potentially pathogenic *E. coli* were present at every stage of the production process and procedures should be put into place to test for these *E. coli* strains to ensure the safety of our meat supply. This was reinforced by the findings of the second study that demonstrated that EHEC with specific virulence genes were detected in almost half of all samples studied at all stages of the production continuum. The presence of EHEC that were positive for the *ehxA* gene in samples obtained during slaughter suggests that analysis of a greater number of samples may result in the isolation of genetically related pathogenic *E. coli* strains from each step in the production process.

One of the limitations of the use of VCCWBA as a selective medium is that it may not select for strains of STEC with the *eaeA* gene but do not carry the *ehxA* gene. Many cases of HUS may go undiagnosed because laboratories only use methods that will isolate strains of *E. coli* O157:H7 and thus miss many potentially virulent strains of STEC (Djordjevic et al., 2001). Methodologies need to be developed to facilitate the rapid isolation of these strains of non-O157:H7 STEC from a variety of samples.

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Appendix A

Animal Numbering System for Lacombe Cattle Herd

Table A-1: Comparison of Lacombe Research Centre animal numbering system and Adam's numbering system used throughout this study.

Original Animal Number	Adam's Numbering System
1156	1
1098	2
1142	3
1105	4
1115	5
1128	6
1103	7
1110	8
1131	9
1107	10
1126	11
1106	12
1114	13
1134	14
1135	15
1147	16
1120	17
1119	18
1139	19
1129	20
1097	21
1138	22
1133	23
1109	24
1160	25
1100	26
1140	27
1118	28
1101	29
1117	30

Appendix B

Characterization of EHEC virulence genes in *E. coli* obtained using VCCWBA from faecal, hide, caecal, and ground beef enrichment broths.

Table B-1: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of faecal samples obtained from animal 5 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
F5-1	-	-	-	+	-
F5-2	+	-	-	-	-
F5-3	-	-	-	-	-
F5-4	+	-	-	-	-
F5-5	-	-	-	+	-
F5-6	+	-	-	-	-
F5-7	+	-	-	-	-
F5-8	+	-	-	-	-
F5-9	+	-	-	-	-
F5-10	+	-	-	-	-
F5-11	+	-	-	-	-
F5-12	+	-	-	-	-
F5-13	-	-	-	-	-
F5-14	-	-	-	-	-
F5-15	+	-	-	-	-
F5-16	+	-	-	-	-
F5-17	+	-	-	-	-
F5-18	+	-	-	-	-
F5-19	+	-	-	-	-
F5-20	-	-	-	-	-
F5-21	+	-	-	-	-
F5-22	+	-	-	-	-
F5-23	+	+	-	-	-
F5-24	+	-	-	-	-
F5-25	+	-	-	-	-
F5-26	-	-	-	-	-
F5-27	+	-	-	-	-
F5-28	+	-	-	-	-
F5-29	+	-	-	-	-
F5-30	-	-	-	+	-
F5-31	-	-	-	+	-
F5-32	+	-	-	-	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Table B-2: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of hide samples obtained from animal 5 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
H5-1	-	+	-	-	-
H5-2	-	-	-	-	-
H5-3	+	+	-	-	-
H5-4	+	+	-	-	-
H5-5	+	+	-	-	-
H5-6	+	-	-	-	-
H5-7	+	-	-	-	-
H5-8	+	+	-	-	-
H5-9	-	-	-	-	-
H5-10	+	+	-	-	-
H5-11	+	+	-	-	-
H5-12	+	+	-	-	-
H5-13	+	+	-	-	-
H5-14	+	+	-	-	-
H5-15	+	-	-	-	-
H5-16	+	+	-	-	+
H5-17	+	+	-	-	+
H5-18	+	+	-	-	+
H5-19	+	+	-	-	+
H5-20	+	+	-	-	+
H5-21	+	+	-	-	+
H5-22	+	-	-	-	-
H5-23	-	-	-	-	-
H5-24	+	+	-	-	+
H5-25	+	+	-	-	+
H5-26	+	+	-	-	+
H5-27	+	+	-	-	+
H5-28	+	+	-	-	+
H5-29	+	+	-	-	+
H5-30	+	+	-	-	+
H5-31	+	+	-	-	+
H5-32	+	+	-	-	+
H5-33	-	-	-	-	-
H5-34	+	+	-	-	+
H5-35	+	+	-	-	+
H5-36	+	+	-	-	+
H5-37	+	+	-	-	+
H5-38	+	-	-	-	-
H5-39	-	+	-	-	-
H5-40	+	+	-	-	+
H5-41	+	+	-	-	+

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Table B-3: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of ground beef samples obtained from animal 5 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
GB5-1	+	-	-	-	-
GB5-2	+	-	-	+	-
GB5-3	-	-	-	-	-
GB5-4	+	-	-	-	-
GB5-5	-	-	-	-	-
GB5-6	-	-	-	-	-
GB5-7	+	-	-	-	-
GB5-8	+	-	-	-	-
GB5-9	+	-	-	-	-
GB5-10	+	+	-	-	-
GB5-11	+	-	-	-	-
GB5-12	+	-	-	-	-
GB5-13	+	-	-	-	-
GB5-14	+	-	-	-	-
GB5-15	+	-	-	-	-
GB5-16	-	-	-	-	-
GB5-17	-	-	-	-	-
GB5-18	-	-	-	-	-
GB5-19	-	-	-	-	-
GB5-20	+	-	-	-	-
GB5-21	-	-	-	-	-
GB5-22	+	-	-	-	-
GB5-23	+	-	-	-	-
GB5-24	+	-	-	-	-
GB5-25	+	-	-	-	-
GB5-26	+	-	-	-	-
GB5-27	+	-	-	-	-
GB5-28	+	-	-	-	-
GB5-29	+	-	-	-	-
GB5-30	+	-	-	-	-
GB5-31	+	-	-	-	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Table B-4: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of faecal samples obtained from animal 10 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
F10-1	+	-	-	-	-
F10-2	-	-	-	-	-
F10-3	+	-	-	-	-
F10-4	+	-	-	-	-
F10-5	+	-	-	-	-
F10-6	+	-	-	-	-
F10-7	+	-	-	-	-
F10-8	+	-	-	-	-
F10-9	+	+	-	-	-
F10-10	+	-	-	-	-
F10-11	+	+	-	-	-
F10-12	+	-	-	-	-
F10-13	-	-	-	-	-
F10-14	+	-	-	-	-
F10-15	+	+	-	-	-
F10-16	+	+	-	-	-
F10-17	+	-	-	-	-
F10-18	+	-	-	-	-
F10-19	+	-	-	-	-
F10-20	-	-	-	-	-
F10-21	+	-	-	-	-
F10-22	+	+	-	-	-
F10-23	+	-	-	-	-
F10-24	+	-	-	-	-
F10-25	+	+	-	-	-
F10-26	+	-	-	-	-
F10-27	-	-	-	-	-
F10-28	-	-	-	-	-
F10-29	+	-	-	-	-
F10-30	+	+	-	-	-
F10-31	+	-	-	-	+
F10-32	+	-	-	-	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Table B-5: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of ground beef samples obtained from animal 10 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
GB10-1	+	-	-	-	-
GB10-2	+	-	-	-	-
GB10-3	+	+	-	-	-
GB10-4	+	-	-	-	-
GB10-5	-	-	-	-	-
GB10-6	+	-	-	-	-
GB10-7	+	-	-	-	-
GB10-8	+	-	-	-	-
GB10-9	-	-	-	+	-
GB10-10	+	-	-	-	-
GB10-11	+	+	-	-	-
GB10-12	-	-	-	-	-
GB10-13	+	+	-	+	-
GB10-14	-	-	-	-	-
GB10-15	+	-	-	-	-
GB10-16	+	+	-	-	-
GB10-17	-	-	-	+	-
GB10-18	-	-	-	+	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Table B-5: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of faecal samples obtained from animal 15 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
F15-1	+	-	-	-	-
F15-2	+	-	-	-	-
F15-3	+	-	-	-	-
F15-4	+	-	-	-	-
F15-5	-	-	-	-	+
F15-6	-	+	-	-	-
F15-7	+	-	-	-	-
F15-8	+	-	-	-	-
F15-9	+	-	-	-	-
F15-10	+	-	-	-	-
F15-11	+	-	-	-	-
F15-12	+	-	-	-	-
F15-13	+	-	-	-	-
F15-14	+	-	-	-	-
F15-15	+	-	-	-	-
F15-16	+	-	-	-	-
F15-17	+	-	-	-	-
F15-18	+	-	-	-	-
F15-19	+	-	-	-	-
F15-20	+	-	-	-	-
F15-21	+	-	-	-	-
F15-22	+	+	-	-	+
F15-23	+	+	-	-	-
F15-24	-	+	-	-	+
F15-25	+	+	-	-	-
F15-26	+	-	-	-	-
F15-27	+	+	-	-	-
F15-28	-	+	-	-	-
F15-29	+	-	-	-	-
F15-30	+	-	-	-	-
F15-31	+	-	-	-	-
F15-32	+	+	-	-	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Table B-7: PCR characterization of *E. coli* strains isolated from the mTSB-n[a] enrichment broth of hide samples obtained from animal 15 that had been plated onto VCCWBA[b].

Isolate #	*E. coli* Marker	EHEC Virulence Genes			
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*
H15-1	-	-	-	-	-
H15-2	+	+	-	-	-
H15-3	+	-	-	-	-
H15-4	+	+	-	-	-
H15-5	+	+	-	+	+
H15-6	+	-	-	-	-
H15-7	-	-	-	-	-
H15-8	+	+	+	-	-
H15-9	+	-	-	-	-
H15-10	-	-	-	+	-
H15-11	+	-	-	-	+
H15-12	+	+	-	-	+
H15-13	+	-	-	-	-
H15-14	-	-	+	-	-
H15-15	+	-	-	-	-
H15-16	+	+	-	-	-
H15-17	+	+	-	-	-
H15-18	+	+	-	-	-
H15-19	+	+	-	-	-
H15-20	+	+	-	-	-
H15-21	+	-	-	-	-
H15-22	-	-	-	-	-
H15-23	-	-	-	-	-
H15-24	+	-	-	-	-
H15-25	-	+	-	-	-
H15-26	+	-	-	-	-
H15-27	+	-	-	-	-
H15-28	+	-	-	-	-

[a] mTSB-n, modified tryptic soy broth supplemented with novobiocin.

[b] VCCWBA, vancomycin, cefixime, cefsulodin washed sheep blood agar.

Appendix C

Identification of EHEC virulence genes in faecal, hide, caecal, and ground beef enrichment broths obtained hroughout the beef cattle production continuum:

Table C-1: EHEC virulence factors and marker genes detected in purified genomic DNA from the faecal samples obtained from cattle after one month on pasture and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx-1*	*stx-2*	*eaeA*	*uidA*	
1	+	-	+	-	-	nd[c]	11
2	+	+	-	-	-	nd	3
3	+	-	-	+	-	nd	6
4	+	-	+	-	-	nd	11
5	+	+	+	-	+	nd	9
6	+	+	+	+	-	-	5
7	+	+	+	-	-	nd	10
8	+	+	+	+	+	-	1
9	+	-	-	+	-	nd	6
10	+	-	-	-	-	nd	0
11	+	-	-	+	-	nd	6
12	+	+	+	+	-	+	5
13	+	+	-	+	-	nd	2
14	+	-	-	+	-	nd	6
15	+	+	-	+	-	-	2
16	+	-	+	+	-	nd	14
17	+	-	+	+	+	-	4
18	+	+	-	+	-	nd	2
19	+	-	-	+	-	nd	6
20	+	-	-	-	-	nd	0
21	+	-	+	-	+	nd	12
22	+	-	-	-	-	nd	0
23	+	-	-	-	-	nd	0
24	+	+	-	+	-	-	2
25	+	+	-	-	-	nd	3
26	+	-	+	-	-	nd	11
27	+	-	+	+	-	-	14
28	+	-	-	+	+	+	15
29	+	-	-	+	-	nd	6
30	+	-	-	+	-	nd	6

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

Table C-2: EHEC virulence factors and marker genes detected in purified genomic DNA from the faecal samples obtained from cattle after five months on pasture and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*Stx*-2	*eaeA*	*uidA*	
1	+	+	-	-	-	nd[c]	3
2	+	-	-	-	-	nd	0
3	+	+	-	-	-	nd	3
4	+	-	-	-	-	nd	0
5	+	+	+	-	-	nd	10
6	+	-	+	-	-	nd	11
7	+	+	-	-	-	nd	3
8	+	-	-	-	-	nd	0
9	+	+	+	-	-	nd	10
10	+	+	-	-	-	nd	3
11	+	+	-	-	-	nd	3
12	+	-	-	-	-	nd	0
13	+	-	-	-	-	nd	0
14	+	-	+	-	-	nd	11
15	+	+	-	-	-	nd	3
16	+	+	-	-	-	nd	3
17	+	+	-	-	-	nd	3
18	+	+	-	-	-	nd	3
19	+	+	-	-	-	nd	3
20	+	-	-	-	-	nd	0
21	+	+	-	-	-	nd	3
22	+	-	-	-	-	nd	0
23	+	-	-	-	-	nd	0
24	+	+	-	-	-	nd	3
25	+	+	+	-	-	nd	10
26	+	-	-	-	-	nd	0
27	+	-	+	-	-	nd	11
28	+	-	-	-	-	nd	0
29	+	+	-	-	-	nd	3
30	+	+	+	-	-	nd	10

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

Table C-3: EHEC virulence factors and marker genes detected in purified genomic DNA from the faecal samples obtained from cattle after one month in feedlot and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*	
1	+	-	-	+	-	nd[c]	6
2	+	+	-	-	-	nd	3
3	+	-	-	-	-	nd	0
4	+	-	-	+	-	nd	6
5	+	-	-	-	-	nd	0
6	+	+	+	+	-	-	5
7	+	-	+	-	-	nd	11
8	+	-	-	-	-	nd	0
9	+	-	-	-	-	nd	0
10	+	-	-	-	-	nd	0
11	+	-	-	-	-	nd	0
12	+	-	+	-	-	nd	11
13	+	-	-	-	-	nd	0
14	+	+	-	-	-	nd	3
15	+	+	-	-	-	nd	3
16	+	-	+	-	-	nd	6
17	+	-	-	-	-	nd	0
18	+	-	+	-	-	nd	6
19	+	-	+	-	-	nd	6
20	+	+	-	-	-	nd	3
21	+	-	-	-	-	nd	0
22	+	-	+	-	-	nd	6
23	+	-	+	-	-	nd	6
24	+	-	-	-	-	nd	0
25	+	+	-	+	-	nd	10
26	+	-	+	-	-	nd	6
27	+	+	-	+	-	nd	10
28	+	-	-	-	-	nd	0
29	+	-	-	-	-	nd	0
30	+	-	-	-	-	nd	0

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

Table C-4: EHEC virulence factors and marker genes detected in purified genomic DNA from the faecal samples obtained from cattle after five months in feedlot and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*	
1	+	-	-	-	-	nd[c]	0
2	+	+	+	-	+	-	9
3	+	-	+	-	+	-	12
4	+	-	+	-	-	nd	11
5	+	-	-	-	+	nd	7
6	+	-	-	+	-	nd	6
7	+	-	-	+	-	nd	6
8	+	-	-	-	-	nd	0
9	+	-	-	+	-	nd	6
10	+	+	-	-	-	nd	3
11	+	-	-	-	-	nd	0
12	+	+	-	+	-	-	2
13	+	-	-	+	-	nd	6
14	+	+	+	-	-	nd	10
15	+	+	-	+	+	+	13
16	+	+	+	+	-	-	5
17	+	-	-	+	-	nd	6
18	+	+	+	+	+	+	1
19	+	+	-	+	-	nd	2
20	+	-	-	-	-	nd	0
21	+	-	-	-	-	nd	0
22	+	-	-	-	-	nd	0
23	+	-	-	-	-	nd	0
24	+	-	-	-	-	nd	0
25	+	+	+	-	-	nd	10
26	+	+	+	+	+	+	1
27	+	-	+	+	-	nd	14
28	+	-	-	+	-	nd	6
29	+	+	+	+	-	-	5
30	+	-	-	-	-	nd	0

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

Table C-5 EHEC virulence factors and marker genes detected in purified genomic DNA from the hide samples obtained from cattle at times of slaughter March 21st (1-15) and April 3rd, (16-30) and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*	
1	+	-	-	+	+	nd[c]	15
2	+	+	+	+	+	-	1
3	+	+	+	-	+	nd	9
4	+	+	+	-	+	nd	9
5	+	+	+	+	+	-	1
6	+	+	+	-	+	nd	9
7	+	+	+	+	+	-	1
8	+	+	+	-	+	nd	9
9	+	-	+	+	+	nd	4
10	+	-	+	+	+	-	4
11	+	+	+	+	-	nd	5
12	+	+	+	+	+	-	1
13	+	+	+	+	+	+	1
14	+	+	+	+	+	+	1
15	+	+	+	+	+	-	1
16	+	-	-	+	-	nd	6
17	+	-	-	+	-	nd	6
18	+	-	-	+	-	nd	6
19	+	-	-	+	-	nd	6
20	+	-	-	+	-	nd	6
21	+	-	-	+	-	nd	6
22	+	-	-	-	-	nd	0
23	+	-	-	+	-	nd	6
24	+	-	-	-	-	nd	0
25	+	-	-	-	-	nd	0
26	+	+	-	-	-	nd	3
27	+	-	-	+	-	nd	6
28	-	-	-	-	-	nd	0
29	+	-	-	-	-	nd	0
30	+	-	-	+	-	nd	6

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

Table C-6: EHEC virulence factors and marker genes detected in purified genomic DNA from the caecal samples obtained from cattle at times of slaughter March 21st (1-15) and April 3rd, (16-30) and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*	
1	+	-	-	-	-	nd[c]	0
2	+	-	+	-	-	nd	11
3	+	+	+	+	+	-	1
4	+	-	-	-	-	nd	0
5	+	+	-	-	+	nd	8
6	+	-	-	-	-	nd	0
7	+	+	-	-	-	nd	3
8	+	+	+	+	+	-	1
9	+	+	-	+	-	nd	2
10	+	+	+	-	-	nd	10
11	+	+	+	+	+	-	1
12	+	+	-	-	+	nd	8
13	+	+	-	-	+	nd	8
14	+	+	-	-	-	nd	3
15	+	+	-	-	+	nd	8
16	+	-	+	-	-	nd	1
17	+	-	-	-	-	nd	0
18	+	+	-	-	-	nd	3
19	+	-	-	+	-	nd	6
20	+	-	-	-	-	nd	0
21	+	-	-	-	-	nd	0
22	+	-	-	-	-	nd	0
23	+	-	-	+	-	nd	6
24	+	-	-	-	-	nd	0
25	+	+	-	-	+	nd	8
26	+	+	-	-	-	nd	3
27	+	+	-	+	-	nd	2
28	+	-	-	-	-	nd	0
29	+	-	-	-	-	nd	0
30	+	+	-	-	+	nd	8

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.

Table C-7: EHEC virulence factors and marker genes detected in purified genomic DNA from the ground beef samples obtained from cattle at times of slaughter March 24th (1-15) and April 6th, (16-30) and enriched in mTSB-n[a].

Animal #	*E. coli* Marker	EHEC Virulence Genes				β-glucuronidase gene	Virulence Profile (VP)[b]
	uspA	*ehxA*	*stx*-1	*stx*-2	*eaeA*	*uidA*	
1	-	nd[c]	nd	nd	nd	nd	0
2	-	nd	nd	nd	nd	nd	0
3	-	nd	nd	nd	nd	nd	0
4	+	+	-	-	+	nd	8
5	+	+	-	-	+	nd	8
6	-	nd	nd	nd	nd	nd	0
7	-	nd	nd	nd	nd	nd	0
8	+	+	-	-	+	nd	8
9	+	-	-	-	-	nd	0
10	+	+	-	-	+	nd	8
11	-	nd	nd	nd	nd	nd	0
12	+	-	-	+	-	nd	6
13	+	-	-	+	-	nd	6
14	-	nd	nd	nd	nd	nd	0
15	+	-	+	-	-	nd	11
16	+	-	-	-	-	nd	0
17	+	-	-	-	-	nd	0
18	+	-	-	-	-	nd	0
19	-	nd	nd	nd	nd	nd	0
20	-	nd	nd	nd	nd	nd	0
21	+	-	-	-	-	nd	0
22	-	nd	nd	nd	nd	nd	0
23	-	nd	nd	nd	nd	nd	0
24	-	nd	nd	nd	nd	nd	0
25	+	-	-	-	-	nd	0
26	-	nd	nd	nd	nd	nd	0
27	-	nd	nd	nd	nd	nd	0
28	-	nd	nd	nd	nd	nd	0
29	-	nd	nd	nd	nd	nd	0
30	-	nd	nd	nd	nd	nd	0

[a] mTSB-n, modified trypic soy broth supplemented with novobiocin.

[b] Virulence profile is the number assigned to indicate specific EHEC virulence genes present in samples from each animal, numbers are assigned as per Table 15.

[c] nd= not determined.